NEW INSIGHTS INTO THE ORGANIZATION AND FUNCTION OF THE EXTRACELLULAR SIGNAL-REGULATED KINASE SIGNALING PATHWAY IN THE PITUTARY GONADOTROPE

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The ERK pathway, a ubiquitously expressed signal transduction module that mediates cellular responses to a variety of stimuli, is activated in gonadotropes following stimulation with the hypothalamic decapeptide GnRH. Several lines of evidence suggest that ERK signaling is important for gonadotrope function; however, little is known regarding the role of ERK signaling in these cells in vivo. The studies described here address aspects of the functional organization and requirement for ERK signaling in differentiated gonadotropes and the role of ERK activity within the reproductive axis in vivo.

ERK 2 associated partially and constitutively with low buoyant density membranes (membrane rafts) following fractionation of both αT3-1 gonadotrope cells and murine pituitaries. Following treatment with GnRH agonist, activated ERKs were recovered in association with low-density membranes. ERK2 coimmunoprecipitated with the GnRHR from these fractions suggesting that coupling of the GnRHR to the ERK signaling pathway may involve the formation of signaling complexes associated with membrane rafts.

To examine the requirement for ERK signaling within gonadotropes in vivo, a mouse model with a pituitary-targeted ablation of ERK1 and ERK2 was generated. ERK deficient male mice were fertile while females demonstrated anovulatory infertility associated with a deficiency of luteinizing hormone (LH). The mechanism

underlying this gender-specific phenotype was linked to impaired transcriptional upregulation of the ERK-dependent transcription factor Egr-1 which is required for LH biosynthesis.

Finally, to explore the role of ERK signaling in upregulation of immediate early response genes in the gonadotrope, we examined expression of the orphan nuclear receptor Nur77. Our results establish Nur77 as an ERK-dependent, GnRH-responsive immediate early gene in the gonadotrope. Analysis of the signaling activities required for Nur77 upregulation led to the unexpected finding that c-Raf kinase is not required for GnRH-induced activation of the ERK pathway. Mice with a pituitary-targeted conditional ablation of c-Raf were viable and fertile. Transcriptional upregulation of Nur77 was unimpaired in gonadotropes from these mice, while being blocked in gonadotropes from mice with pituitary disruption of ERK1 and 2.

These results confirm the requirement for ERK signaling for gonadotrope function, and shed new light on the organization of this pathway in the gonadotrope as well as its role in the sexually dimorphic regulation of reproductive function.

BIOGRAPHICAL SKETCH

Stuart Bliss was born in 1964 in Ithaca, New York. His secondary education took place in both the mid-Hudson Valley in New York, and Brussels, Belgium. He attended Bates College in Lewiston, Maine from 1982 through 1984, and after a brief hiatus from higher education, completed a BA degree from the State University of New York at Albany in 1988 with majors in philosophy and music. He then completed a post-baccalaureate premedical basic science curriculum at the same University from 1990 through 1991, and entered the College of Veterinary Medicine at Cornell University in 1992. He earned the DVM degree from Cornell in 1996. From 1996 through 2001 he completed a one-year rotating clinical internship at the College of Veterinary Medicine at North Carolina State University, three years of residency training in small animal surgery at the College of Veterinary Medicine at Cornell University, and a one-year fellowship in small animal clinical orthopedics, also at Cornell. Stuart earned board certification from the American College of Veterinary Surgeons in 2003.

In 2001 Stuart matriculated in the Program in Comparative Medicine at Cornell University, joining the laboratory of Dr. Mark Roberson in early 2002.

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LIST OF ABBREVIATIONS

GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
GPKR	G protein-coupled receptor kinase
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	MAP kinase ERK kinase
MKP	MAP kinase phosphatase
RTK	Receptor tyrosine kinase
ERK	Extracellular Signal-regulated kinase
RSK	Ribosomal S6 kinase
KSR	Kinase suppressor of ras
JNK	Jun-n-terminal kinase
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
αGSU	Glycoprotein hormone alpha subunit
cAMP	Cyclic-adenosine monophosphate
ACTH	Adrenocorticotropic hormone
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
BMP	Bone morphogenic protein

FGF	Fibroblast growth factor
EGF	Epidermal growth factor
NGF	Nerve growth factor
SF1	Steroidogenic factor 1
IP_3	Inositol triphosphate
DAG	Diacylglycerol
PLC	Phospholipase
PKA	Protein kinase A
PKC	Protein kinase C
BAR	Beta-adrenergic receptor
VGCC	Voltage-gated calcium channel
IGF	Insulin-like growth factor
DRM	Detergent-resistant membranes
PLAP	Placental alkaline phosphatase
MHC	Major histocompatability complex
TCR	T-cell receptor
MBCD	Methyl-beta-cyclodextrin
FRET	Fluorescence resonant energy transfer
ESR	Electron spin resonance
ER	Endoplasmic reticulum
CD	Common docking
MMP	Matrix metalloproteinase
ADAM	Alpha-disintegrin and transmembrane metalloproteinase

CHAPTER 1

BACKGROUND AND REVIEW OF THE LITERATURE

1. Introduction:

In mammals, reproductive competence depends on the coordinated activity of an intercommunicating set of endocrine tissues and organs referred to as the hypothalamic-pituitary gonadal (HPG) axis. The gonadotrope cell of the anterior pituitary plays a particularly critical role within this system as the intermediary between the hypothalamic GnRH signal and the germ cell reservoirs and steroid hormone productivity of the gonads. The mechanisms underlying the response of the gonadotrope to hypothalamic GnRH are thus of critical importance to our understanding of the molecular basis of reproductive function.

Expression of the GnRH receptor is a defining characteristic of the gonadotrope. Binding of GnRH to its receptor triggers a complex array of intracellular signal transduction events within the gonadotrope; these signaling cascades orchestrate the overall physiological response of these cells to GnRH stimulation. The extracellular-signal regulated kinase (ERK) signaling pathway is a conserved signal transduction module that is strongly activated in gonadotropes following GnRH stimulation. ERK signaling is generally understood to be important for gonadotrope function; however, much remains to be learned regarding the mechanisms and consequences of GnRH-induced ERK activation in these cells, especially within the intact endocrine milieu of the living animal.

2. The mammalian hypothalamic-pituitary-gonadal (HPG) axis

In the classical view, the HPG axis is depicted as a tiered and linearly organized set of endocrine tissues that is dedicated to the regulation and support of reproductive activity (1). This axis consists of a subset of hypothalamic neurons that express the decapeptide hormone gonadotropin-releasing hormone (GnRH), the gonadotrope cells of the anterior pituitary, and the gonads. Activation of this

endocrine axis commences with the pulsatile secretion of GnRH from the hypothalamus. GnRH is delivered to the anterior pituitary at high concentration via the hypophyseal portal circulation where it binds to GnRH receptors (GnRHR) on the surface of gonadotropes triggering the synthesis and secretion of the gonadotropins follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The gonadotropins are dimeric glycoprotein hormones composed of distinct β subunits paired with a common α subunit (αGSU). The genes for FSHβ and LHβ, together with the αGSU and the GnRHR comprise the genetic signature of the differentiated gonadotrope. In the male, LH stimulates production of testosterone and androgen-binding protein by testicular Leydig cells and Sertoli cells, respectively. In the female, LH stimulates the production of androgens by the thecal cells that surround the growing ovarian follicle. FSH binds to receptors on the surface of ovarian granulosa cells stimulating the expression of aromatase enzymes that convert thecal androgens to estradiol. During the terminal stages of follicular growth, LH also drives the production of progesterone from the granulosa cells of the preovulatory follicle (2).

Several animal models have provided insights into the functional organization of the HPG axis. The hypogonadal (HPG) mouse fails to produce GnRH due to a deletion mutation in the gene encoding the GnRH precursor peptide (3). HPG mice never enter puberty and display a persistent hypogonadotropic hypogonadal phenotype which is nevertheless able to be rescued by knockin of a functional GnRH gene (4). Early studies in sheep showed that surgical ablation of the pituitary stalk (hypothalamic-pituitary disconnection, HPD) led to dramatic decreases in pituitary secretion of the gonadotropins and secondary hypogonadism (5, 6). Pulsatile administration of GnRH to these animals reestablished appropriate pulsatile gonadtropin secretion and fertility while continuous administration of GnRH led to virtual cessation of pituitary gonadotropin production (7).

In mice, elimination of the gene for either LHβ, or the LH receptor (LHR), leads to infertility in both genders associated with marked decreases in gonadal steroid hormone production, along with defective spermatogenesis and late follicular developmental arrest (8, 9). FSH levels are normal in these mice and the hypogonadal phenotype is able to be rescued by exogenous LH. In contrast, female mice lacking the gene for FSHβ are infertile due to a defect in ovarian follicular maturation; however, males remain fertile, despite decreased testicular size and sperm production (10). Similarly, the phenotype of female mice lacking the FSH receptor (FSHR) is similar to that of the FSHβ-deficient animal (infertility due to follicular developmental arrest) (11, 12). However, in males, loss of the FSHR leads to a greater impairment of gonadal function than in FSHβ nulls, with significant decreases in Leydig cell numbers and levels of circulating testosterone (13). It has been suggested that the more severe phenotype displayed by FSHR deficient animals as compared with those lacking FSH itself, is indicative of constitutive and physiologically important ligand-independent activity of gonadal FSHR populations (13).

In addition to information gained from animal models, many naturally-occuring human diseases provide insight into the HPG axis function. Kallmann Syndrome (KS) is a form of hypogonadotropic hypogonadism that results from a failure of migration of GnRH neurons from the olfactory epithelium to the hypothalamus during development and the consequent lack of hypothalamic GnRH production (14). Individuals with KS typically are anosmic (deficient in the sense of smell) due to hypoplasia or aplasia of the olfactory tracts and in addition may display a variety of developmental abnormalities including dental or renal agenesis, palate defects, digit malformations and hearing loss (15). Several specific mutations have been identified that collectively account for approximately 30% of reported cases of KS. The most severe form of the disease (KAL1) is an X chromosome-linked

condition associated with a loss-of-function mutation in the *KAL1* gene that encodes the protein anosmin-1 (14, 16). Anosmin-1 is a secreted multidomain glycoprotein that is thought to guide developing axonal projections during morphogenesis of the olfactory system in part through stabilization of growth factor-receptor complexes, especially complexes between fibroblast growth factors (FGFs) and their receptors (FGFRs) (17, 18). Since olfactory axonal tracts function as a pathway along which nascent GnRH neurons migrate from their origin in the olfactory epithelium to the hypothalamus, disturbances in the development of the olfactory system may interfere with the ability of GnRH neurons to populate the hypothalamus. This leads to GnRH deficiency and hypogonadotropic hypogonadism. Loss-of-function mutations in *FGFR1* have also been associated with an autosomal dominant form of KS (KAL2); however this form of the disease is incompletely penetrant, indicating a more complex and probably oligogenic basis (19, 20).

Mutations in the gene encoding the human GnRHR have been discovered that lead to hypogonadotropic hypogonadism of variable severity (21). It has been shown that some mutations of the GnRHR lead to misfolding and abnormal intracellular trafficking of the newly synthesized protein that results in failure of receptor expression (22). This is discussed in more detail in section 4.1 below. Inactivating mutations in the genes for LHβ and FSHβ as well as their receptors have also been reported. These mutations are generally associated with hypogonadal phenotypes, and given their adverse effects on reproductive function, are understandably rare (21).

2.1 Hypothalamic secretion of GnRH

The release of GnRH from the hypothalamus is inherently pulsatile, and this pulsatility is required for appropriate gonadotropin production by the pituitary (23). Both the amplitude and frequency of GnRH pulses are tightly regulated and, in the

female, vary dramatically over the course of the reproductive cycle (23). Rapid GnRH pulsatility has been shown to promote synthesis and secretion of LH, while slower GnRH pulsatility favors production and release of FSH (24). The corresponding oscillations in plasma levels of the gonadotropins may further reflect the shorter circulatory half-life of LH as compared to FSH.

The importance of GnRH pulsatility was first demonstrated in 1978 in experiments in Rhesus monkeys with surgically created lesions of the basomedial hypothalamus that eliminated the GnRH neurons (25). Continuous administration of exogenous GnRH to these animals led to decreased production of gonadotropins and hypogonadism, while pulsed administration of GnRH at hourly intervals restored gonadal function and reproductive cyclicity (26). The suppression of pituitary responsiveness to GnRH following continuous exposure is now known to be caused by downregulation of GnRHR's at the level of the gonadotrope, although the precise mechanism by which this downregulation occurs remains unclear. Downregulation of GnRHR's by continuous GnRH exposure forms the basis for the clinical use of long-acting GnRH analogs that are prescribed during infertility treatments (27).

2.2 Feedback regulatory mechanisms in the HPG axis

The HPG axis is subject to both positive and negative feedback regulation at several levels. At the level of the hypothalamus, early recognition of the pulsatile nature of GnRH secretion led to the notion of a central "pulse generator", the inherent oscillatory activity of which controls the secretory rhythm of GnRH neurons (28). Anatomically, this pulse generator is located predominantly within the anteroventral paraventricular nucleus (AVPV) of the hypothalamus. Interestingly, studies from dispersed hypothalamic neurons in primary culture suggest that pulsatile secretion of GnRH may be an inherent property of the neurons themselves (29). Moreover,

populations of GnRH neurons may be capable of broad scale synchronization of GnRH pulsatility, and this synchronization does not appear to require direct intercellular contact (30). GnRH neurons express the GnRHR, which couples to different intracellular heterotrimeric G-proteins in a manner dependent on ligand dose (31). These observations have led to a simplified model in which low doses of GnRH may exert positive autocrine and paracrine feedback effects on neuronal GnRH secretion through GnRHR-$G\alpha_s$-induced increases in intracellular cyclic adenosine-monophosphate (cAMP) levels. The resulting increase in GnRH secretion subsequently propels dramatic further positive feedback effects by driving GnRHR-$G\alpha_{q/11}$-induced increases in intracellular calcium. However, once the concentration of extracellular GnRH reaches a sufficiently high (threshold) level, GnRHR-$G\alpha_i$-mediated signaling pathways are preferentially activated, leading to negative feedback suppression of secretory activity (32).

Regardless of the extent to which oscillatory release of GnRH is an inherent property of the GnRH neurons, it is clear that the hypothalamic pulse generator is modulated by a multitude of inputs, in particular androgens and estrogens (33). In the male, testosterone exerts negative feedback effects primarily on the release of GnRH from the hypothalamus (34). A minor negative feedback effect may also be exerted at the level of the gonadotrope (34). In the female, the feedback effects of gonadal steroids on HPG function are more complex and depend on the stage of the reproductive cycle. During the follicular phase, estrogen downregulates both the pulse frequency and pulse amplitude of GnRH release from the hypothalamus, through a direct inhibitory effect on GnRH neurons (35). This leads primarily to suppression of LH production by the gonadotrope (36). During this phase estrogen also downregulates LH production through direct effects on the gonadotrope. However, during the preovulatory period, the effects of estrogen on both the hypothalamus and

the pituitary become positive (37). This positive effect of estrogen on GnRH production is indirect, mediated by afferent estrogen-sensitive neurons of the AVPV that synapse on the GnRH neurons (35). This leads to rapid and significant increases in hypothalamic GnRH secretion culminating in the massive preovulatory release of LH from the pituitary (LH surge) that is the definitive trigger of ovulation.

The predominant negative inhibitory effects of gonadal steroids on hypothalamic GnRH production provide a useful means for manipulation of the hypothalamic-pituitary system in experimental animals. For example, removal of the gonads upregulates GnRH production by the hypothalamus due to the disinhibitory effects of decreased gonadal steroid levels (38, 39). Gonadectomy thus subjects the gonadotrope to a physiologically appropriate pulsatile GnRH hyperstimulation in vivo, an effect that is impossible to recapitulate within a cell culture system. This strategy was employed in the studies described in Chapter 3 of this dissertation.

3. Developmental background of the gonadotrope

The anterior lobe of the pituitary is composed of five discrete hormone producing cell types; in addition to the gonadotrope, the corticotrope, thyrotrope, somatotrope, and lactotrope cells respond to distinct hypothalamic releasing factors and produce adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL), respectively. During embyrogenesis, these hormone-producing cell lineages develop from a common pituitary primordium in a highly regulated spatial and temporal progression. The distinct phenotypes expressed by these cells vis-a-vis their hormone production has made the pituitary a useful model system in which to study basic mechanisms of cellular differentiation and organogenesis in vivo. A comprehensive review of pituitary development is beyond the scope of this dissertation; however, several basic

concepts related to the development of pituitary cell types are integral to the hypotheses and experimental approaches described below in chapters 3 and 4, and are therefore outlined here in brief. Pituitary development is reviewed in detail in ref. (40).

3.1 Basic mechanisms underlying pituitary morphogenesis

The pituitary is an ectodermal derivative that arises from the most anterior portion of the cranial placodes at the midline region of the neural plate. Expansion and bending of the forebrain structures early in development repositions this placodal ectoderm to the roof of the oral cavity. At e8.5 in the mouse, this ectodermal region undergoes a dorsal invagination to form Rathke's pouch, the anatomical precursor of the anterior and intermediate lobes of the pituitary. The ectodermal region destined to undergo invagination is marked by a lack of expression of the morphogen sonic hedgehog (shh) which is otherwise expressed throughout the surrounding ectoderm. Simultaneously, a ventral outgrowth of the overlying diencephalon occurs (the infundibulum) which is destined to become the posterior pituitary. Expression of bone morphogenic protein 4 (BMP4) by the adjacent overlying region of diencephalon has been shown to be a critical inductive stimulus for the initial invagination of Rathke's pouch, and close contact between Rathke's pouch and the infundibulum is necessary for subsequent morphogenesis of the gland.

Shortly after the appearance of Rathke's pouch, diencephalic expression of members of the fibroblast growth factor (Fgf) family, specifically Fgf's 8, 10, and 18 commences, while diencephalic production of BMP4 subsides. Concurrently, BMP2 begins to be expressed at the boundary region between Rathke's pouch and the surrounding oral ectoderm. The LIM homeodomain transcription factors Lhx3, Lhx4, and Isl1 are also expressed within the pouch at this time where they function to

maintain pouch cells in an undifferentiated state while contributing to the ensuing proliferative expansion of the pituitary primordium and final separation of the pouch from the oral ectoderm. These dorso-ventral gradients of diencephalic Fgf signals, combined with shh and BMP2 from the oral ectoderm and adjacent pouch region, provide important morphogenic cues that guide expansion and shaping of the gland. FGF8 is of particular importance in this process. Experiments using explants of Rathke's pouch in primary culture indicate that Fgf8 alone is able to recapitulate the effects of the entire diencephalic infundibular region with respect to maintenance of Lhx expression, repression of Isl1 expression, and pouch cell proliferation (41). It thus stands to reason that the intracellular signal transduction cascades that mediate the cellular effects of Fgf's are critical to the process of early pituitary morphogenesis.

Proliferative expansion of the pituitary primordium and sequential appearance of lineage-committed cell types of the anterior portion of the gland occurs from e9.5 through approximately e13.5. These processes are dependent upon tightly regulated spatiotemporal expression of numerous transcription factors (42). The αGSU is the first hormonal subunit gene to be expressed within the developing pituitary, appearing in the ventral region of Rathke's pouch at e10.5 (40). The distribution and timing of αGSU promoter activation has been studied in transgenic mice using reporters linked to various portions of the αGSU promoter. A 381 base pair fragment of the murine αGSU promoter is sufficient to direct gonadotrope and thyrotrope specific reporter expression within the developing pituitary; however, higher levels of expression are obtained with the use of a 4.6 kb promoter region that incorporates an upstream enhancer element (43). One study using this 4.6 kb αGSU promoter fragment showed reporter expression as early as e9.5 throughout the pituitary primordium (44). The possibility of such early, promiscuous, or misregulated activation of this transgenic promoter fragment during development is important in relation to the studies described

in chapters 3 and 4 below, where in vivo genetic recombination was accomplished using this same 4.6 kb promoter to regulate expression of Cre recombinase.

3.2 Developmental specification and differentiation of the gonadotrope lineage

The developmental events underlying fate specification of the gonadotrope lineage remain largely unclear. This is in contrast to other pituitary lineages such as the thyrotrope, somatotrope and lactotrope cells (the Pit-1 lineages) whose development and differentiation are dependent on expression of the POU domain containing transcription factor Pit-1 (45). The orphan nuclear receptor steroidogenic factor-1 (SF1) is first expressed at e13.5 and is the first gonadotrope-specific marker to appear within the developing gland (46). Onset of expression of SF1 is occasionally cited at the defining event in gonadotrope specification. Indeed, pituitary specific depletion of SF1 led to impaired expression of the gonadotropins as well as the GnRHR, and secondary hypogonadism (47). However, this phenotype was able to be rescued by administration of exogenous GnRH suggesting that while SF1 is necessary for gonadotrope function, it is not required for developmental specification or differentiation of the gonadotrope lineage (47).

The gonadotrope is the last of the anterior pituitary cell types to undergo terminal differentiation as marked by the relatively late onset of expression of the genes encoding the β-subunits of FSH and LH. The mechanisms underlying the timing of initial expression of these gonadotropin subunit genes are unknown. Activation of the murine FSHβ promoter first occurs at approximately e17.5 while LHβ biosynthesis commences approximately 24 hours later. In conjunction with a cohort of relatively ubiquitous and cell type-*non*-specific transcription factors, SF1 plays a key role in activation of the promoters for all of the genes that comprise the genetic signature of the gonadotrope (αGSU, FSHβ, LHβ, GnRHR), and is primarily

responsible for restricting expression of FSHβ, LHβ, as well as GnRHR to the gonadotrope (48).

4. Molecular basis of the response of the gonadotrope to GnRH

4.1 Experimental models used in the study of gonadotrope function

Studies of GnRH-activated signal transduction pathways in the gonadotrope have relied heavily on the αT3-1 and LβT2 murine gonadotrope-derived cell lines. These cell lines were generated through targeted expression of SV40 large T antigen in developing pituitary cells (49-51). Both lines express the GnRHR as well as the αGSU. In addition, the LβT2 cell line expresses the β subunit of LH, and may thus represent a somewhat more differentiated gonadotrope phenotype. For the studies described in Chapters 2 and 4 below, αT3-1 cells were used exclusively.

Protocols for purification of gonadotropes from dispersed pituitary tissue and their propagation in primary culture have been described; however they are technically demanding and are not widely used (52). However, due to the restricted pattern of expression of the GnRHR as well as the specificity of GnRH, many aspects of gonadotrope function, particularly transcriptional responses to GnRH stimulation, can be readily examined in primary cultures of mixed pituitary cells generated by enzymatic dispersal of fresh pituitary tissue. Measurements of GnRH-induced changes in transcript levels in primary culture systems must be interpreted with caution, since expression of a gene of interest in cell types other than the gonadotrope will contribute to baseline transcript levels within an experimental unit, and will lead to underestimation of the effects of hormone stimulation on the subpopulation of gonadotropes within that unit. This is relevant to the results described in Chapter 3 where GnRH-induced increases in mRNA levels of Egr1, a transcription factor known

to be expressed in multiple pituitary cell types other than gonadotropes, were measured in mixed primary pituitary cultures.

Finally, many transgenic models have been developed that shed light on the role of specific gene products within the pituitary. Constitutive gene ablation may result in a phenotype that reflects primary pituitary dysfunction. For example, despite its broad pattern of expression, mice lacking Egr1 display female infertility associated with a failure of LH production in the gonadotrope (53). Alternatively, due to their more restricted pattern of expression, the promoters of genes encoding a number of the pituitary trophic hormones have been used to target transgene expression to the pituitary allowing conditional, Cre recombinase-mediated disruption of floxed alleles (54-56). This approach is described in Chapters 3 and 4 where the αGSU promoter was used to drive pituitary targeted expression of Cre recombinase, and subsequent Cre-mediated disruption of the ERK2 and Raf-1 genes.

4.2 Structural and functional aspects of the GnRH receptor

The GnRHR is a member of the large G-protein coupled receptor (GPCR) superfamily. Three forms of the GnRHR have been identified in vertebrates, designated types I, II, and III (57). Three corresponding forms of GnRH (type I, II, and III) have also been identified (57). The type I receptor is the predominant form expressed in the gonadotrope, and in some mammalian species including humans is also expressed in certain extra-pituitary tissues most notably the breast, gonads, prostate, and uterus (58). The natural ligand for the type I receptor is type I GnRH; however type II GnRH may also engage and activate the type I receptor (59). Indeed, in several mammalian species, including human and mouse, the type II GnRHR has been evolutionarily silenced at the genomic level by a frameshift mutation. Thus, the

biological effects of GnRH II or its analogues are thought to be mediated by the type I receptor (60).

The type I GnRHR was first cloned in 1992 from cDNA derived from the murine αT3-1 gonadotrope-derived cell line (61, 62). Subsequent cloning of GnRHR's from human, rat, sheep, pig, and horse showed that the receptor is highly conserved, with greater than 80% amino acid homology among these mammalian species (63). The deduced amino acid sequence predicts a 327 amino acid (aa) receptor with seven transmembrane domains, a 35 aa amino-terminal extracellular domain with 2 putative glycosylation sites, and a conspicuously short 1-2 aa carboxyl-terminal cytoplasmic domain. An additional glycosylation site is predicted within the first extracellular loop (62, 63). While the crystal structure of the GnRHR has not been determined, its homology with other members of the GPCR family, including rhodopsin, has allowed model-based predictions of the overall topology of the receptor, as well as the structural basis of its interaction with ligands (64). Many of these predictions have been verified by site-directed mutagenesis studies in recombinant receptors expressed in cell culture systems. For example, alanine substitution of asparagine residues 18 or 102 (predicted glycosylation sites) led to decreased receptor glycosylation, and while this had no effect on ligand affinity, overall receptor expression was decreased, indicating a role for glycosylation of these residues in receptor stability or appropriate membrane targeting (65). Similar methods have identified critical residues involved in ligand binding within the second and seventh transmembrane domains, as well as the third extracellular loop (66-68) . Insight into the structural basis of the interaction of the receptor with its decapaptide ligand has facilitated the development of receptor antagonists, as well as high-affinity ligands that function as super-agonists (65). Antide and buserelin (des-GLY^{10} [D-$Ser(t\text{-}But)^{6}$]-LH-RH Ethylamide) are examples of GnRHR antagonist, and super-

agonist, respectively, that are commonly used in molecular and cellular studies of gonadotrope function. Both compounds were used in the investigations described here.

The lack of a carboxyl-terminal intracellular domain renders the mammalian type I GnRHR a structurally and functionally unique member of the GPCR family (63). The C-terminal tail domain of the prototypical GPCR is an important target for phosphorylation, typically mediated by a G-protein coupled receptor kinase (GRK) (69). C-terminal tail phosphorylation generates a docking site for members of the β-arrestin family of scaffolding proteins, which upon binding, mediate rapid desensitization and dynamin-dependent internalization of a receptor via clathrin-coated pits (70). Lack of a C-terminal tail implies that the GnRHR may be resistant to rapid desensitization and internalization, and this has been confirmed experimentally for this receptor in a variety of settings (71-74). Domain swap studies in which the C-terminal tail of the thyrotropin-releasing hormone (TRH) receptor was fused to the C-terminus of the mammalian GnRHR rendered the receptor susceptible to rapid arrestin-mediated desensitization and internalization (75). Exposure of gonadotropes to GnRH does lead to receptor internalization; however, this process occurs more slowly than with other GPCR's, and appears to be independent of dynamin (76). Indeed, the GnRHR has been described as a "naturally-occuring internalization deficient mutant" GPCR (77).

Appropriate plasma membrane expression of the GnRHR is of obvious importance for responsiveness of the gonadotrope to GnRH. In turn, surface expression of functional receptors requires proper intracellular folding, glycosylation, and trafficking of newly synthesized receptor proteins. Naturally-occuring mutations have been described in the human GnRHR gene that lead to hypogonadotropic hypogonadism of variable clinical severity (21). These mutations have been mapped

to various locations throughout the length of the GnRHR coding sequence. Functional analysis of recombinant receptors harboring many of these mutations indicates that ligand binding and coupling to intracellular effectors are unimpaired, but that the mutant receptors undergo improper folding and either aberrant intracellular trafficking or premature degradation (22). Loss of newly synthesized receptors due to stochastic processes of misfolding and premature degradation is common for some membrane receptors, and underscores the dynamic nature of receptor biosynthesis and recycling (78, 79). In many instances, cell permeable ligand-mimetics may shift the equilibrium of this process in favor of effective receptor expression by improving the efficiency with which nascent receptors adopt a proper conformation during folding (80, 81). This has been shown for the GnRHR where treatment of cells with the GnRH-mimetic indole IN3 was able to establish GnRH responsiveness in cells expressing a variety of mutant receptors that otherwise underwent premature degradation (22). This has stimulated interest in the mechanisms underlying appropriate plasma membrane targeting of the GnRHR as well as the development of pharmacological agents that would allow manipulation of cell surface receptor density by altering the efficiency of folding and trafficking.

4.3 GnRH-induced signal transduction in the gonadotrope

The signaling events initiated by engagement of the GnRHR have been the subject of considerable investigation (82-85, 95, 97, 175, 176, 184). As with other GPCR's, ligand binding induces a conformational change in the receptor that leads to dissociation and activation of a heterotrimeric G-protein generally involving the $G\alpha_{q/11}$ subunit (85). GTP loading of $G\alpha_{q/11}$ leads to activation of phospholipase $C\beta$, and subsequent elaboration of the second messengers, phophatidylinositol-3-phosphate (IP_3) and diacylglycerol (DAG). DAG leads to activation of PKC isozymes

and contributes to a sharp rise in intracellular calcium concentration, which derives from both IP_3-mediated release of intracellular calcium stores, as well as influx of extracellular calcium through L-type voltage gated channels (84). These events are prerequisite for activation of mitogen-activated protein kinase (MAPK) activity, in particular the extracellular signal-regulated kinase (ERK) pathway (83).

4.3.1 Membrane-associated signaling events

The delayed kinetics of GnRHR internalization underscore the importance of the plasma membrane as a platform for organization of the early signaling events that occur following ligand binding. This is in contrast to other GPCRs such as the β-adrenergic receptor (βAR) that undergo rapid internalization and that may nucleate active signaling complexes on the surface of endosomes (86).

The GnRHR has been shown to couple to multiple G-proteins depending on cell type and experimental model; however, the predominant signaling events intiated by GnRHR occupancy reflect the activation of $G\alpha_{q/11}$ family members (85). $G\alpha_{q/11}$ comprises a group of pertussis toxin-insensitive palmitoylated GTP-binding proteins that lead to selective activation of the β−isoforms of phospholipase C.

Detailed structural and functional analysis of protein domains within PLC-β indicate that these enzymes undergo constitutive and reversible membrane association mediated by the C-terminal region as well as the N-terminal plekstrin homology domain (87). Activation of PLCβ does not involve membrane recruitment. Catalytic activity of PLCβ is stimulated by interaction with GTP-loaded $G\alpha_{q/11}$ subunits, and leads to the elaboration of IP_3 and DAG within the plasma membrane. PLCβ also acts as a $G\alpha_{q/11}$ GTPase activating protein (GAP) leading to negative feedback effects on the signaling input by stimulating hydrolysis of $G\alpha_{q/11}$ bound GTP (88). PLCβ isoforms are also present within the nucleus, where they catalyze a nuclear cycle of

inositol-phosphate (IP) turnover that has been shown to be important in cellular responses to various mitogenic stimuli (89). Interestingly, nuclear PLCβ1 is a direct ERK substrate, and ERK-mediated phosphorylation of S982 within the C-terminal domain is required for PLCβ1 activation by Insulin-like Growth Factor 1 (IGF-1) (90). Of note, S982 resides within a regulatory region of the C-terminal domain that is also required for interaction with $G\alpha_{q/11}$ (91). Whether nuclear IP turnover plays any role in GnRH action, or whether GnRH-activated PLC isoforms are regulated by phosphorylation at the level of the plasma membrane is unknown.

In contrast to PLCβ, activation of PKC isoforms involves plasma membrane recruitment. Three major classes of PKC have been characterized, namely the conventional, novel, and atypical PKCs (92). All are activated through the synergistic effects of DAG and phosphatidylserine. In addition, activation of the conventional PKCs requires calcium. αT3-1 cells express a variety of PKCs representing all classes including the α, β, ε, θ, and ζ isoforms (93, 94). Fractionation experiments indicated that the α, ε, and ζ isoforms may be the major PKCs activated by GnRH in these cells (94). The phorbol ester PMA is a potent activator of all classes of PKC. Similarly, the pharmacological agent GF109203X (GFX) is a commonly used inhibitor of PKC activity that is active against all classes.

In αT3-1 cells, PKC activity is required for ERK pathway activation by GnRH; however the mechanism underlying this requirement is unknown (95). Raf-1 kinase is widely believed to be the key upstream activator of the ERK signaling module in the gonadotrope, and direct activation of Raf-1 by PKC has been suggested as a mechanism by which GnRH-induced signaling events at the plasma membrane may link to an ERK cascade that ultimately converges on the nucleus (96, 97). Membrane recruitment is a critical step in the complex series of events that leads to Raf-1 activation; thus, recruitment and activation of Raf-1 by membrane-bound PKC

is an conceptually attractive scenario. However, little evidence exists to support direct activation of Raf-1 by PKC. Furthermore, the data presented in Chapter 4 raise significant doubt regarding the importance of Raf-1 itself in GnRH-induced ERK activation, either in αT3-1 cells or in vivo. The architecture of this portion of the GnRH signal transduction network, particularly the link between signaling activities at the plasma membrane and the ERK module, thus remain unclear.

4.3.2 Membrane rafts and the GnRHR

The membrane raft hypothesis

The fluid mosaic model of cellular membrane organization, originally proposed by Singer and Nicholson in 1972, describes the plasma membrane of cells as a homogenous fluid phase lipid bilayer containing numerous peripheral and integral membrane proteins, in which individual lipid molecules are essentially unconstrained with respect to their ability to diffuse laterally through the membrane (98). Subsequent investigations into the properties of model membranes composed of binary and ternary mixtures of lipids at defined molar ratios, as well as cellular membranes, has led to a contrasting model of the plasma membrane as a heterogeneous assembly of spatially and functionally distinct domains that may differ with respect to both lipid and protein composition (99-101). This membrane raft hypothesis is predicated upon the notion that different lipid species within a membrane bilayer may differ in their affinities for each other and for membrane proteins, and that these differences in affinity may facilitate the formation of biochemically and biophysically distinct lipid domains within the membrane.

Definition of the membrane raft

Early descriptions of membrane rafts (often referred to as lipid rafts) were based on the observation that glycosylphosphtidyl-inositol (GPI)-anchored proteins remained insoluble when cells were homogenized at low temperature in the presence of the non-ionic detergent Triton X-100, and were consistently recovered in association with low-bouyant density membranes following sucrose density gradient centrifugation of cell homogenates (102). Subsequent analysis of the composition of these detergent-resistant membranes (DRM's) showed that they were enriched in cholesterol and sphingolipids as well as numerous specific integral membrane proteins (103). From these early characterizations of DRM's emerged the notion that the plasma membrane may be organized as a lipid bilayer containing discrete cholesterol and sphingolipid-enriched microdomains (lipid rafts) that serve as platforms for the recruitment of specific membrane proteins. Perhaps due to the relative ease with which DRM's may be prepared from a variety of cell types, a largely operational definition of membrane rafts seems rapidly to have gained popularity in many areas of cell biology. Indeed, hundreds of reports on the role of membrane rafts in various cellular contexts were published in ensuing years, many of which seem based on the assumption that DRM's provide a biologically meaningful representation of membrane domains as they exist within the plasma membrane of living cells. As a caution against uncritical acceptance of this assumption, Lai proposed a definition of the membrane raft as "an unidentified floating object" (104). Nevertheless, acceptance of this assumption appears to remain widespread.

A recent Keystone Symposium on Lipid Rafts and Cell Function was held at which the following definition of membrane rafts was proposed: "Membrane rafts are small (10-200 nm) heterogeneous, highly dynamic, sterol and sphingolipid enriched domains that compartmentalize cellular processes" (105).

Phase separation within model and cellular membranes

The temperature-dependent formation of biochemically and biophysically distinct lipid domains within model membranes composed of simple lipid mixtures is well established. For example, when membranes composed of binary mixtures of lipids are brought to temperatures intermediate between the melting points of the individual lipid species, the lipids with the higher melting point may spontaneously aggregate into domains of solid phase within a surrounding milieu of fluid phase, or liquid disordered (ld), lipid (106). In simple lipid mixtures, this phase separation is not abrupt, but occurs continuously over a range of temperatures (106). Addition of cholesterol to binary lipid mixtures has been shown to broaden the range of temperature over which phase separation occurs following thermal fluctuations and to lead to the formation of a distinct lipid phase referred to the liquid ordered (lo) phase (107). In the lo phase the acyl chains of individual lipid molecules assume an extended, closely packed, and highly ordered conformation akin to a solid phase, but retain high lateral mobility within the plane of the membrane, as in an ld phase (107). Cholesterol has been shown to be critical for the formation of the lo phase. The rigid planar configuration of cholesterol may facilitate the extension of adjacent acyl chains and close lipid packing (108). Cholesterol may also associate preferentially with sphingolipids, the large polar protruding head groups of which may help shield the hydrophobic ring structure of cholesterol from the surrounding aqueous environment (umbrella effect) (109). Hydogen bonding between cholesterol and sphingolipids may also contribute to the nonuniform distribution of cholesterol throughout the membrane and the formation of cholesterol enriched lo domains (110).

Large scale phase separation of persistent lo lipid domains may form in response to temperature changes, even in more complex vesicular membranes derived from the plasma membrane of living cells (111). However, smaller and more transient

nanodomains have also been identified in model membranes of more complex chemical composition at physiologically appropriate temperatures (112). These observations suggest that while phase separation and formation of lo domains are likely to occur in cellular membranes, both their size and the time scales over which they exist are likely to be extremely small. Indeed it has been proposed that the definition of the membrane raft be broadened to include highly transient molecular assemblies involving as few as 3 molecules with lifetimes of tens of milliseconds (113).

Properties and functions of membrane rafts

The tight packing of lipids within lo domains creates a unique membrane environment for which integral and peripheral membrane proteins may show highly different affinities. Rafts thus provide a theoretical mechanism for regulation of protein-protein interactions at the level of the plasma membrane through the partitioning of individual proteins into, or out of, regions of lo phase.

While the tendency of certain acylated cytoplasmic proteins, or GPI-linked exoplasmic proteins, to partition efficiently into regions of lo phase would seem predictable, experimental observations indicate that this often occurs only weakly. For example, when the GPI anchored protein placental alkaline phosphatase (PLAP) was introduced into unilamellar vesicles containing coexisting lo and ld phases, the reporter protein actually showed preferential partitioning into regions of ld phase (114). This occurred despite its reported tendency to partition into low-bouyant density DRM following cold detergent extraction (102). However, antibody crosslinking of the PLAP markedly shifted its behavior within the membrane, leading to its preferential association with lo domains (114). Similarly, in homogeneous model membranes containing the ganglioside GM1, large scale segregation of lo

membrane domains was induced in the absence of thermal fluctuations by external crosslinking of GM1 (115). These observations are consistent with notion that membrane rafts may generally be extremely small and short-lived, but that specific extracellular stimuli that lead to crosslinking or clustering of membrane proteins may induce the formation of large scale and more persistent lo domains that in turn could act as platforms for the recruitment of specific proteins or the assembly of specific protein complexes (113).

Perhaps the most substantial evidence in support of this general hypothesis comes from studies of T-lymphocyte activation. Use of the phase sensitive fluorescent lipid reporter laurdan indicated that regions of lo phase are small and inconspicuous in non-stimulated T cells (116). Occupancy of the T cell receptor (TCR) with antigen-MHC complex together with CD28 costimulation (the basic signals that drive T cell activation) leads to clustering of TCR-CD3-CD28 complexes. This receptor clustering is thought to be the driving force behind the regional condensation of plasma membrane around activated receptors into an lo phase, leading ultimately to the formation of a large membrane domain enriched in TCR and associated signaling complexes (the distal component of the immunological synapse). This regional membrane condensation was evidenced by a phase-dependent shift in laurdan fluorescence at sites of TCR activation (116). Overall these observations point to a finely regulated interplay between lo membrane nano-domains, specific extracellular signals that may induce coalescence of these nanodomains into larger regions of lo phase through clustering or crosslinking of receptors, and raft associated recruitment of specific signaling complexes that may assemble in a stimulus-dependent manner in the vicinity of activated receptors.

Controversies surrounding the role of membrane rafts in biological systems

Despite the large body of published work focused on the role of membrane rafts in various cell types, controversy continues to surround many aspects of the membrane raft hypothesis, including the size, lifespan, composition, function, and even the existence, of raft domains (104, 117). Much of this controversy appears to reflect the technical difficulty involved in either isolation of raft domains from the plasma membrane of living cells or direct visualization of rafts in situ.

Several methods for purification of raft domains from cells have been described; most involve density gradient centrifugation of cell lysates prepared by extraction and mechanical homogenization of cells in the presence of low concentrations of a non-ionic detergent such as Triton X-100 (118). Detergent-free methods have also been described by which cellular membranes may be fractionated on the basis of density alone (119, 120). As with the results described in Chapter 2 below, the association of specific proteins with low density membrane fractions is the fundamental readout for these experiments, and the tendency of a given protein to compartmentalize within a microdomain of the plasma membrane of a living cell is inferred therefrom. These techniques are relatively simple and are commonly used; however they have several limitations. Phase separation in membranes is highly influenced by temperature. Nevertheless, standard fractionation techniques are typically performed at 4^{o}C, with little regard for the ability of cold temperatures to induce membrane condensation or even protein precipitation. Moreover, nonionic detergents have been shown to induce artifactual micron-scale segregation of domains from cellular membranes at low temperatures (121). Parallel use of detergent-free fractionation methodologies may be warranted as a control for detergent artifacts.

An additional significant limitation of subcellular fractionation for study of membrane rafts is lack of temporal and spatial resolution. Results of fractionation

studies may provide useful information regarding the general preference or affinity of a protein for a specific membrane environment. However, from the point of view of membrane dynamics, these techniques are incapable of disclosing the time scales over which rafts may form and disperse in cells, nor can they capture the residence time or the rate of trafficking of a protein into or out of a membrane domain (113). Similarly, fractionation methods generally yield micron scale fragments of protein-laden cellular membranes. The resemblance of such large scale membrane condensations to the nanometer scale domains proposed to exist in situ is debatable.

The formation of lo phase within membranes is dependent on cholesterol. Manipulation of cellular cholesterol levels has therefore become a widely used technique for assessing, at least indirectly, the association of a protein with raft domains. Depletion of membrane cholesterol perturbs membrane domain organization, and loss of association of a protein with DRM or low density membranes following cholesterol depletion is often cited in support of that protein's inherent affinity for a lo membrane environment (122). Cholesterol depletion is typically accomplished by exposure of cells to the compound methyl-β cyclodextrin (MBCD). MBCD is a cell-impermeable surface acting carbohydrate that efficiently extracts cholesterol from lipid bilayers by sequestration in a hydrophobic pocket. Cholesterol depletion by MBCD is reversible through the exposure of cells to MBCD that has been presaturated with cholesterol, allowing for acute membrane cholesterol repletion, or over-repletion. MBCD has become a commonly used tool for probing the function of membrane rafts in living cells; however, inference regarding the behavior or function of rafts based on cholesterol depletion experiments is also problematic (122). Cholesterol comprises approximately 30% of plasma membrane lipid on a molar basis, and up to 90% of total cellular cholesterol is contained within the plasma membrane (123). While enriched within raft domains, cholesterol is also present within bulk ld

regions of plasma membrane and is susceptible to MBCD extraction from these regions as well as from rafts (122). Cholesterol depletion may thus induce extensive structural and functional perturbations of the plasma membrane. Acute cholesterol depletion is known to increase membrane permeability; indeed in our hands, exposure of αT3-1 cells to MBCD led to rapid but reversible permeabilization within minutes as assessed by Trypan Blue staining (S. Bliss, unpublished data). MBCD has also been shown to bind to phospholipids and may facilitate exchange of phospholipids between preexisting membrane domains (124). Thus, while cholesterol depletion may be informative regarding the raft association of specific proteins, assessment of its effects on coordinated cellular activities such as signal transduction that may be sensitive to changes in overall cell membrane integrity clearly must be interpreted with caution.

In lieu of biochemical approaches, numerous sophisticated biophysical techniques have been developed in recent years for direct characterization of membrane rafts in situ. These include confocal microscopic imaging of cells treated with phase-sensitive membrane reporters, FRET analysis of protein or lipid juxtapositions within cellular or model membranes, single particle tracking, and high temporal resolution electron spin resonance (ESR) spectroscopic methods for evaluation of the residency time of lipids within specific domains (125-128). These techniques have been instrumental in the development of the current view of membrane rafts as small and highly dynamic membrane heterogeneities that may nevertheless play important roles in signal transduction, especially through ligand-dependent clustering and stabilization.

Membrane rafts and the GnRHR

Previously, the GnRHR was shown to partition completely into low-bouyant density membrane fractions following sucrose density gradient centrifugation of αT3-

1 cell homogenates prepared both in the presence and absence of detergent, suggesting that the receptor may have an inherent preference for a lo membrane environment (129). Following cholesterol depletion, cold detergent extraction solubilized the receptor; however, its association with low-density membranes was restored by cholesterol repletion. Cholesterol depletion also blocked the ability of GnRH to activate the ERK pathway while ERK responsiveness to phorbol-ester induced activation of PKC was maintained. Analysis of inositol phosphate production indicated that cholesterol depletion uncoupled the GnRHR-G-protein complex from PLCβ. This blockade in signaling activity was also restored by cholesterol repletion (129). ERK activation was also blocked by increasing cellular cholesterol levels to 200% of baseline through exposure of cells to cholesterol-loaded MBCD without initial depletion (S Bliss, unpublished data). These observations led to the hypothesis that the ability of the GnRHR to signal appropriately is affected by the biophysical properties of the plasma membrane, and may require association with lo plasma membrane microdomains (membrane rafts).

FRET studies with fluorochrome labeled GnRHR showed that agonist treatment led to self-association (dimerization) of receptors and decreased lateral mobility of receptors within the membrane (130). Receptor dimerization also occurred following treatment with antide; however, this receptor antagonist had no effect on lateral mobility of the receptor (130). This suggests that agonist-induced receptor clustering may facilitate coupling of the receptor with the downstream intracellular signaling apparatus. Consistent with this hypothesis, several key signaling intermediates known or presumed to play a role in the GnRHR-ERK pathway were also found to associate at least partially with low density membranes including $G\alpha_{q/11}$, calmodulin, isoforms of the 14-3-3 family of adapter proteins, Raf-1, MEKs, and the ERKs themselves (129, 131). The experiments described in Chapter 2

below were undertaken to examine in greater detail the association of ERKs with GnRHR-enriched plasma membrane microdomains and to further test the hypothesis that GnRH-induced ERK activation involves the assembly of a membrane-associated multiprotein signaling complex in proximity to the GnRHR.

4.3.3 Calcium signaling in the gonadotrope

In the gonadotrope, GnRH induces a rapid biphasic elevation of intracellular calcium (132). The initial sharp rise in cytosolic calcium reflects predominantly the release of calcium from intracellular storage depots, particularly the endoplasmic reticulum (ER). Following this initial spike, a more sustained phase of calcium elevation occurs, due primarily to influx of extracellular calcium via L-type voltage gated channels (VGCC). Release of intracellular calcium from the ER is triggered by IP_3 through direct interaction with IP_3 receptors on the surface of the ER. Calcium influx through VGCC is dependent on the activity of PKC isozymes.

Calcium influx via VGCC has been shown to be uniquely required for ERK activation in the gonadotrope, although the mechanisms underlying this requirement remain unclear (83, 133). In previous work from this laboratory, GnRH stimulation led to rapid calcium loading of the calcium sensing protein calmodulin, which was itself shown to be required for ERK activation independently of the calmodulin-dependent protein kinase CamKII (131). In vitro reconstitution experiments indicated direct calcium-dependent interaction between calmodulin and Raf-1 (131). In addition, calmodulin was also recovered from low-density DRM following sucrose density gradient centrifugation of αT3-1 cell homogenates, suggesting that calmodulin may serve as a link between VGCC calcium and the ERK pathway, and that this linkage may be regulated through association with membrane rafts (131). The cellular effects of calcium signals have long been known to be highly compartmentalized

(microdomain calcium signaling) in a wide variety of excitable and nonexcitable cells (134). Thus, while inconclusive, our observations raise the intriguing possibility that the contribution of VGCC calcium to GnRH-induced ERK activation in the gonadotrope represents a similarly compartmentalized event, possibly organized through association with plasma membrane rafts.

Recent studies have identified proline-rich tyrosine kinase 2 (Pyk2) as a calcium-dependent enzyme required for GnRH-induced ERK activation in both αT3-1 and LβT2 cells. In LβT2 cells, Pyk2 was shown to act as a scaffold for the assembly of a signaling complex containing c-Src, Grb2, and Sos, and this complex was suggested to form a direct link between GnRH-induced calcium currents and canonical Ras-dependent activation of the ERK pathway (135). Recent work from this laboratory also identified Pyk2 as a catalytic activity required for GnRH-induced ERK activation in αT3-1 cells and showed that GnRH induces rapid phosphorylation of Pyk2 in mouse gonadotropes in vivo (136). In agreement with previous results demonstrating a requirement for calmodulin for GnRH-induced ERK activation, this study documented a direct interaction between calcium-activated calmodulin and the catalytic domain of Pyk2 (131, 136). Calmodulin was further shown to be required for GnRH-induced Pyk2 phosporylation in mouse gondotropes in vivo. Pyk2 thus appears to play a key role in linking VGCC-mediated calcium signals with the ERK pathway in gondotropes.

4.3.4 GnRH-induced MAP kinase activation

The mitogen-activated protein kinase (MAPK) pathways are a highly conserved set of signal transduction cascades that mediate cellular responses to an enormous variety of environmental stimuli. In mammals, there are 4 predominant MAPK pathways: the ERK, jun-N-terminal kinase (JNK), p38, and ERK5/Big MAP

kinase (ERK/BMK) pathways. The basic organization of these pathways is similar, consisting of a multi-level phosphotransfer system. Activation of the pathway begins with phosphorylation of an upstream MAP kinase-kinase kinase, which phosphorylates and activates an intermediate level MAP kinase kinase, which in turn phosphorylates and activates the terminal MAP kinase. Activated MAP kinases phosphorylate numerous substrates throughout the cell including elements of the transcriptional machinery, chromatin components, cytoskeletal structures, and other downstream enzymes (137, 138).

In the gonadotrope, GnRH stimulation leads to activation of the ERK, JNK and p38 pathways (84). Several studies link activation of these pathways to transcriptional regulation of the gonadotropin genes. The role of ERK5/BMK in the gonadotrope has not been investigated.

5. The ERK signaling pathway

The ERK signaling pathway is the most intensively studied and thoroughly characterized of the MAPK systems. In its canonical form, the core element of the ERK pathway consists of a 3-level kinase cascade including the MAPKKK Raf-1, the MAPKK's MEK1 and 2, and the MAPK's ERK1 and 2 (139). In addition to these core kinases, numerous scaffolding and adaptor proteins have been shown to play important roles in the functional organization of this pathway (140).

The ERK pathway is involved in the transduction of signals emanating from a multitude of different cell surface receptors. The best characterized of these are the receptor tyrosine kinases (RTKs). Indeed, the ERK proteins were initially identified as kinase activities induced in cells by RTK ligands including a variety of mitogenic peptides and insulin (141, 142). These ligands stimulate the ERK pathway through a classical mechanism involving activation of the low molecular weight GTPase Ras.

The ability of ERKs to mediate the proliferative effects of RTK ligands has been demonstrated in a variety of settings, and has been the focus of intense investigation in the areas of basic cell biology as well as drug discovery and anti-cancer therapeutics (143, 144). The ERK pathway is also activated following engagement of many GPCR (145). Agonists for these receptors include a wide variety of small molecules as well as numerous neurotransmitters, peptides hormones, cytokines, nucleotides and amino acids, ions, and lipids (146). GPCRs are linked to a bewildering array of cellular functions. While the ERK pathway has been shown to be important in mediating the mitogenic effects of many GPCR ligands, ERKs also play a role in the specific responses of some highly differentiated cells to certain agonists (146, 147).

The ability of the ERK pathway to elicit dramatically diverse biological responses in different contexts points to complex and varied mechanisms of signal interpretation that have evolved in cells. Early insights into ERK signal specificity came from studies of the PC12 rat pheochromocytoma cell line in which it was shown that sustained ERK activation induced by Nerve Growth Factor (NGF) led to neuronal differentiation while transient ERK activation following Epidermal Growth Factor (EGF) treatment elicited a non-differentiating proliferative response (148). One established mechanism by which cells may interpret differences in ERK signal duration emerged from studies of the ERK-dependent immediate early response protein c-Fos. As a component of the dimeric transcription factor AP-1, c-Fos is capable of coupling ERK pathway activity with the expression of a wide variety of genes. c-Fos is rapidly upregulated following ERK activation; however, in the absence of post-translational modification, the newly synthesized protein undergoes rapid degradation. ERK-dependent phosphorylation of the C-terminus of c-Fos stabilizes the protein, prolonging its half-life and allowing its nuclear accumulation (149). C-terminal phosphorylation also targets the protein for secondary ERK-

dependent phosphorylation of internal residues 325 and 331, which enhances its transcriptional activation function (149). However, this only occurs under conditions of relatively sustained ERK activation. These dual ERK-dependent phosphorylation events that sequentially stabilize and activate c-Fos provide an example of a simple but elegant mechanism by which cells may link ERK signals of variable duration with different transcriptional outputs.

Protein-protein interactions mediated through defined docking domains are also important in the establishment of ERK signal specificity. ERKs contain 2 key interaction domains: the common docking (CD) domain on the face of the protein opposite the catalytic site, and a separate interaction domain at the edge of the activation site (150, 151). The former binds to cognate 'D' domains of interactors such as ribosomal S6 kinases (RSK), MEK1/2, and certain MAP kinase phosphatases (MKPs). The latter binds to the 'docking for ERK [Phe-X-Phe]' (DEF) domain of many ERK substrates including c-Fos and Egr1(150, 151). Docking interactions are prerequisite for appropriate substrate recognition by ERKs and also guide the interaction of ERKs with various scaffolding and adaptor proteins. These interactions may contribute to signal specificity by confining ERK activity to particular subcellular compartments. For example, docking interactions underlie the nuclear translocation of activated ERKs that occurs in response to various stimuli (152, 153). The MAPKKs MEK 1 and 2 can play an important role in this process. The MEK proteins contain a nuclear export sequence that restricts them to the cytosol (154, 155). Under resting conditions, stable associations between MEKs and inactive ERKs mediated through D domain-CD domain interactions may tether ERKs within the cytosolic compartment (154). Upon pathway activation, however, the MEK-ERK association is destabilized allowing dissociation and nuclear translocation of active ERK.

In addition to their established tendency to undergo nuclear translocation, ERKs may also be recruited to extranuclear membranes, and may be activated within the context of membrane bound signaling complexes (156, 157). The DEF domain containing protein kinase suppressor of ras (KSR) is a scaffold protein that has been shown to facilitate Ras-dependent activation of membrane targeted ERKs through nucleation of signaling complexes containing all the core kinases of the ERK pathway (158). KSR has also been shown to bind Gβγ dimers and to facilitate Ras-independent activation of membrane-associated ERKs following occupancy of certain GPCRs (159). Docking interactions also underlie the organization of ERK signaling complexes by β−arrestins following desensitization and internalization of GPCRs (160). Some data suggest that arrestin-associated complexes may serve to restrict activated ERKs to the cytosolic compartment thereby selectively targeting extranuclear substrates (160, 161). Similarly, formation of focal adhesions may involve the recruitment, activation, and retention of ERKs at sites of adhesion through interactions with p125-FAK or Pyk2 (162). The activity of this pool of ERKs may be restricted to highly compartmentalized phosphorylation of cytoskeletal components of the adhesion complex.

5.1 Mechanisms of ERK activation by GPCR's

In the classical paradigm of GPCR signaling, ligand binding stabilizes the receptor in an active conformation leading to nucleotide exchange of its G-protein α subunit and the dissociation of the α subunit as well as the dimeric βγ subunit from the receptor. The free α subunit then regulates the activity of various enzymatic effectors involved in the production of second messengers (163). Historically, studies of GPCR signaling have emphasized the role of these receptors in highly differentiated cellular functions such as neurotransmission and excitation-contraction coupling. However, in

recent years, appreciation of the role of GPCRs in cellular growth control has led to an interest in the mechanisms by which these receptors may activate established mitogenic pathways, in particular the ERK pathway (146).

The mechanisms of ERK activation by GPCRs vary considerably with both receptor and cell type. In some settings, GPCRs have been shown to cause phosphorylation and activation of RTKs such as the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR) in a process referred to as transactivation (164). The most widely cited mechanism of RTK transactivation is based on the phenomenon of ectodomain shedding. According to this model, GPCR ligands lead to activation of ADAM family transmembrane matrix metalloproteinases (MMPs) through the activity of various intracellular effectors such as calcium, reactive oxygen species, Gβγ dimers, PKC isoforms, and the non-receptor tyrosine kinases c-src and Pyk2. MMPs in turn catalyze the proteolytic release of membrane bound RTK ligands leading to conventional ligand-dependent "outside-in" activation of RTKs and canonical Ras-dependent activation of the Raf-MEK-ERK cascade (165, 166). GPCRs may also drive ERK activation through second messenger-dependent enzyme systems. For example, in the HEK293 cell model, activation of ERK through the endogenously expressed β(2)-adrenergic receptor was shown to involve cAMP-dependent activation of PKA, and subsequent activation of B-Raf via the small Ras-family GTPase Rap-1 (167). β-arrestins have been shown to be capable of linking numerous GPCRs to the ERK module through nucleation of signaling complexes containing internalized receptors along with all the core components of the ERK pathway (160). Finally, Gβγ dimers may couple to the ERK pathway independent of their cognate α-subunit through the direct or indirect activation of the activity of phosphatidylinositol-3-kinase (PI3-kinase) or PLCβ (168).

5.2 Mechanisms of GnRH-induced ERK activation in the gonadotrope

In gonadotropes, GnRH stimulation leads to relatively transient activation of ERKs (84, 169). Studies on the mechanism of GnRH-induced ERK activation in gonadotropes have yielded conflicting results depending on the model systems employed. Nevertheless, some generalizations seem valid. Unlike other GPCRs, the GnRHR is not phosphorylated following ligand binding and the lack of a C-terminal tail precludes direct interaction with β-arrestins (72, 170). Therefore arrestin-mediated mechanisms of ERK activation are unlikely to apply. In contrast, the requirement for extracellular calcium influx and activation of PKC isoforms for ERK activation has been thoroughly demonstrated (84, 133). Previous work from this laboratory showed further that GnRH-induced ERK activation was blocked by inhibition of calmodulin (131). Direct activation of Raf-1 by PKC is often cited as mechanism by GnRH may drive ERK pathway activation in a Ras-independent manner. However, this hypothesis derives largely from a study in which PKC was shown to phosphorylate Raf-1 in vitro; in light of the complex nature of Raf-1 activation, this hypothesis may be largely discounted (96). Moreover, while Raf-1 is widely assumed to be the predominant upstream activator of the ERK pathway in these cells, experimental evidence in support of this is lacking. Current views highlight the role of Raf-1 as an important regulator of apoptosis that is not required for ERK activation in all settings, and suggest that the alternative isoform B-raf may be of greater overall importance as a MAPKKK than Raf-1 (171). Indeed, within the pituitary, activation of the ERK pathway in both corticotrope and somatotrope cells following stimulation with the GPCR ligands corticotropin-releasing hormone (CRH) or growth hormone-releasing hormone (GHRH) does not involve Raf-1, but proceeds through a pathway involving $G\alpha_s$, PKA, Rap-1 and B-raf (172, 173).

One report demonstrated the importance of EGFR transactivation through ectodomain shedding for GnRH-induced ERK activation in αT3-1 cells (174). However, this may reflect a unique property of the cell clones used in those experiments as we have been unable to demonstrate EGF responsiveness in αT3-1 cells at any dose (MS Roberson & S Bliss, unpublished data). Similarly, c-Src has been implicated as a link between the GnRHR and the ERK pathway through the activation of Ras (135, 175). However, other reports indicate that GnRH-induced ERK activation is Ras-independent, and we have been unable to demonstrate phosphorylation of c-Src in αT3-1 cells following exposure to GnRH (MS Roberson, unpublished data) (176). Thus, the mechanisms by which the GnRHR couples to the ERK module remain unclear. The molecular architecture of this region of GnRH signaling cascade would therefore seem to be a promising area of investigation.

5.3 Importance of ERK signaling for gonadotrope function

In gonadotropes, a substantial fraction of active ERK undergoes rapid nuclear translocation where it plays established roles in GnRH-induced transcriptional responses (169). The immediate early gene response to GnRH stimulation was examined by microarray analysis in the LβT2 cell line where 28 immediate early genes were shown to be significantly upregulated within 60 minutes of GnRH exposure (177). The role of ERK signaling in the induction of several of these genes, including c-Fos, Egr1, and LRG21/ATF3, is established (169, 178, 179). As a component of the dimeric transcription factor AP-1, c-Fos has been implicated in GnRH-induced expression of FSHβ, as well as in homologous upregulation of the GnRHR (180, 181). Egr1 plays an established role in the transcriptional upregulation of LHβ, and also contributes to the expression of the dual specificity phosphatase MKP2/DUSP4 (182, 183). ERKs have also been shown to contribute to GnRH-

induced transcriptional upregulation of the αGSU through putative phosphorylation of Ets family transcription factors associated with the αGSU promoter (184). ATF3 may also enhance expression of at least the human αGSU through dimerization with c-Jun (178). ERK signaling is thus linked to the expression of the full complement of genes (GnRHR, FSHβ, LHβ, αGSU) that comprise the genetic signature of the gonadotrope. Whether ERK signaling contributes to the expression of other members of this immediate early gene repertoire in the gonadotrope remains to be determined. The studies described in Chapter 4 below specifically address the role of the ERK pathway in mediating rapid GnRH-induced upregulation of the nuclear orphan receptor Nur77 in the αT3-1 cell line as well as in vivo.

6. Summary and study aims

The mammalian reproductive axis is a finely regulated physiological system in which the gonadotrope plays a keys role. The gonadotrope response to the oscillatory hypothalamic GnRH signal is mediated by the GnRH receptor, and interpretation of this stimulus involves the coordination of multiple intracellular signaling cascades. Recent evidence suggests that the biophysical properties of the plasma membrane may impact GnRH signaling by regulating interaction of the GnRHR with downstream signaling intermediates. The ERK pathway plays an important role in the response of the gonadotrope to GnRH by mediating rapid transcriptional upregulation of a repertoire of immediate early response genes. However, the differential roles of ERK1 and ERK2 in this context have not been addressed. Moreover, the role of ERK signaling in the gonadotrope has been overwhelmingly examined in cell culture models, the physiological relevance of which has not been clearly established. The studies described here had 3 major objectives: (1) to assess the association of ERKs with membrane rafts in gonadotropes, and to determine whether this association is of

regulatory significance for GnRH-induced ERK activation, (2) to define the requirement for ERK signaling for gonadotrope function in vivo using a genetic model, and (3) to examine the role of ERK signaling in mediating GnRH-induced expression of the immediate early response gene Nur77 in gonadotropes.

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CHAPTER 2

SIGNALING COMPLEXES ASSOCIATED WITH THE TYPE I GnRH RECEPTOR: COLOCALIZATION OF ERK2 AND GnRH RECEPTOR WITHIN MEMBRANE RAFTS*

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SUMMARY

Our previous work demonstrated that the type I GnRH receptor resides exclusively and constitutively within membrane rafts in αT3-1 gonadotropes, and that this association was necessary for the ability of the receptor to couple to the ERK signaling pathway. $G_{\alpha q}$, c-raf, and calmodulin have also been shown to reside in this compartment, implicating a raft-associated multiprotein signaling complex as a functional link between the GnRH receptor and ERK signaling. In the studies reported here, we used subcellular fractionation and co-immunoprecipitation to analyze the behavior of ERKs with respect to this putative signaling platform. ERK 2 associated partially and constitutively with low-density membranes both in αT3-1 cells and in whole mouse pituitary. Cholesterol depletion of αT3-1 cells reversibly blocked the association of both the GnRH receptor and ERKs with low-density membranes and uncoupled the ability of GnRH to activate ERK. Analysis of the kinetics of recovery of ERK inducibility following cholesterol normalization supported the conclusion that re-establishment of the association of the GnRH receptor and ERKs with the membrane raft compartment was not sufficient for reconstitution of signaling activity. In αT3-1 cells, the GnRH receptor and ERK2 co-immunoprecipitated from low-density membrane fractions prepared either in the presence or absence of detergent. The GnRH receptor also partitioned into low-density, detergent-resistant membrane fractions in mouse pituitary, and co-immunoprecipitated with ERK2 from these fractions. Collectively, these data support a model in which coupling of the GnRH receptor to the ERK pathway in gonadotropes involves the assembly of a multiprotein signaling complex in association with specialized microdomains of the plasma membrane.

INTRODUCTION

The complex signaling events initiated by engagement of the mammalian type I GnRHR (subsequently referred to as GnRHR) have been the subject of much investigation. In gonadotropes, the GnRH-R couples to $G\alpha_{q/11}$, leading to activation of phospholipase Cβ, and subsequent elaboration of the second messengers, phophatidylinositol-3-phosphate (IP_3) and diacylglycerol (DAG)(1). DAG-dependent activation of PKC isozymes contribute to a sharp rise in intracellular calcium concentration, which derives from both IP_3-mediated release of intracellular calcium stores, as well as influx of extracellular calcium through L-type voltage gated channels (2, 3). GnRH also induces rapid activation of the mitogen activated protein kinase (MAPK) pathways, of which the extracellular signal-regulated kinase (ERK) pathway has been most thoroughly characterized. In gonadotropes, activation of the ERK pathway has been linked to the expression of a complement of immediate early genes such as c-fos (4, 5), ATF-3 (6), and Per-1 (7), as well as several genes essential for gonadotrope function including the glycoprotein hormone α subunit (α-GSU) (8), the LHβ subunit (5, 9), and a regulatory MAP kinase phosphatase (MKP2) (10). Several requirements for ERK activation by GnRH have been defined, including influx of extracellular calcium through L-type voltage gated calcium channels (VGCC) (11), activation of PKC isozymes (12, 13), and calmodulin (14). Nevertheless, the precise mechanism by which the GnRHR couples to the ERK pathway remains unclear.

An important mechanism for regulation of signaling activity that has received widespread attention in recent years involves compartmentalization of signaling molecules through association with specialized low-density membrane microdomains termed membrane rafts (15-17). These domains are cholesterol- and sphingolipid-rich regions of membrane that appear in many cell types to function as platforms for the assembly of multiprotein signaling complexes (18). Rafts are enriched in lipid species

with highly saturated acyl chains, particularly glycosphingolipids and sphingomyelin (19). Interactions between sphingolipids and cholesterol contribute to the formation of a liquid-ordered (l_o) phase. The l_o phase is described as a localized lipid environment in which the acyl chains of individual lipid molecules assume an extended, closely packed, and highly ordered conformation akin to a gel phase, but in which the lipids retain high lateral mobility within the plane of the membrane, as in a fluid phase (20). Liquid-ordered domains present a unique membrane environment for which integral and peripheral membrane proteins may show highly different affinities. These domains thus provide a mechanism for regulation of protein-protein interactions through the partitioning of individual proteins into, or out of, l_o regions.

We have previously reported that the GnRHR resides constitutively and exclusively within a detergent-resistant, low-density membrane environment in αT3-1 gonadotrope cells (21). Cholesterol depletion shifted the receptor into a detergent-soluble non-raft environment, and led to a reversible blockade of ERK activation by GnRH. The GTP-binding protein $G_{\alpha q/11}$, as well as c-raf, the putative upstream activator of the ERK pathway in these cells, co-localized with the receptor in low-density membrane fractions suggesting that these membrane domains may be of functional significance in coupling the GnRHR to components of the ERK pathway (21). To further explore this model, we now report the use of subcellular fractionation techniques to examine the behavior of the ERK proteins with respect to association with low-density membrane domains. We find that ERKs 1 and 2 associate with low-density domains of the plasma membrane in conjunction with the GnRHR in a constitutive and cholesterol-dependent manner, both in αT3-1 gonadotropes, as well as in whole mouse pituitary. Membrane-associated ERKs are activated rapidly following exposure of cells to a GnRH agonist. In addition, the GnRHR and ERKs co-immunoprecipitate from low-density membrane preparations, lending further support

for our model of a raft-associated multiprotein signaling complex as a functional link between the GnRHR and the ERK signaling module.

MATERIAL AND METHODS

Cells, Antibodies, and Chemicals

αT3-1 cells, an immortalized mouse gonadotrope cell line (generously provided by Dr. Pamela Mellon, University of California, San Diego), were cultured as described previously (21). Anti-ERK2 (sc-154-G), anti-$G\alpha_{q/11}$ (sc-352), and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies and FITC-streptavidin used for immunohistochemistry were from Jackson Immunoresearch (West Grove, PA). Anti-phospho-ERK1/2 antibody was from Sigma Chemical Co. (St. Louis, MO). Anti-Flotillin-1 antibody was from BD Biosciences (San Jose, CA). Anti-EEA-1 antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-Ca pan α-1 antibody was from Alomone Labs (Jerusalem, Israel). Anti-LH antibody was from A.C. Parlow and the National Hormone and Peptide Program sponsored by NIDDK. Anti-transferrin receptor antibody was purchased from Zymed laboratories (San Francisco, CA). Tissue-Tech OCT compound was from Miles Inc. (Elkhart, IN). HRP-conjugated streptavidin was from Vector Laboratories (Burlingame, CA). The GnRHR antibody was raised in a rabbit against 20 amino acids of the second extracellular loop (aa193-aa212) of the ovine GnRHR. This sequence shows no overlap with any other receptor or peptide. Buserelin (des-GLY^{10} [D-$Ser(t\text{-}But)^{6}$]-LH-RH Ethylamide; referred to as GnRHa), methyl-β-cyclodextrin (CD), and cholesterol-loaded CD (CDChol), and all other chemicals were obtained from Sigma. In all experiments, GnRHa was used at 10 nM. Alexa Fluor® 594 membrane raft

labeling kit (CTxB) was obtained through Molecular Probes (Eugene, OR). Glass bottom microwell dishes for confocal studies were obtained from Mat-Tek (Ashland, MA). Superfect transfection reagent was purchased from Qiagen (Valencia, CA).

Confocal microscopy

αT3-1 cells grown on glass bottom microwell dishes were transiently transfected with a GnRHR construct with a GFP fluorophore linked to the C-terminus for 48 hours. Following 48 hours of transfection, GM_1 domains were labeled with Alexa Fluor® 594 CTxB at 4°C. Anti-CTxB antibody was then used to crosslink the CTxB labeled domains. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then imaged. Imaging was done utilizing the 63X oil objective and the 488nm and 543nm laser lines of a Zeiss LSM 510 Meta confocal microscope. The Alexa594 and GFP signals were acquired using multitrack mode and no crosstalk between Alexa594 and GFP was observed. Transferrin receptor (Tf-R) co-localization with GM_1 was assessed in αT3-1 cells using the same Alexa Fluor® 594 CTxB staining approach described above. Following GM_1 labeling, αT3-1 cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were blocked in PBS containing 3% BSA and then incubated with anti-transferrin antibody overnight at 4°C. The following day, cells were washed and labeled with an Alexa Fluor® 488 conjugated anti-mouse secondary antibody for 1 hour. Cells were then imaged by confocal laser scanning microscopy (CLSM) as above. Quantitative analysis of fluorophore co-localization is expressed as a scatter diagram (50). Identical images (co-localization) produce a clean diagonal line running from the bottom left to the top right. Differences between the images cause an irregular distribution in the scatter diagram.

Tissue Preparation and Immunohistochemistry

Male mice were anesthetized with ketamine and transcardially perfused with 100ml 1% sodium nitrite in 0.9% NaCl, followed by 200ml of fixative (4% paraformaldehyde, 15% saturated picric acid in 0.1M phosphate buffer (PB), pH 7.4) and then 100ml cryoprotectant (20% sucrose in 0.1M PB). Pituitaries were removed and stored in cryoprotectant overnight at 4^{o}C. Tissue was embedded in Tissue-Tech OCT compound and frozen by immersion in liquid nitrogen-cooled isopentane. Sections (20µm) were mounted on Silane-coated slides and stored at -80C.

Sections were washed (all washes 3×3 min in 0.01M PBS) and incubated (48 h) in a solution containing a rabbit anti-GnRHR antibody (1:2000; 10% goat serum, 0.3% Triton in PBS) as well as a horse anti-ovine LH antibody (1:5000). Sections were washed and immersed in biotinylated goat anti-rabbit IgG and goat anti-horse IgG linked to Texas Red (90 min; both at 1:200). Following additional washing, sections were immersed in FITC-streptavidin (90 min; 1:200), washed, mounted and examined by conventional immunofluoresence microscopy.

Cell Surface Biotinylation

Cell surface biotinylation was performed using a commercial kit (Molecular Probes/Invitrogen, Carlsbad, CA) with minor modifications. Biotin-XX-sulfosuccinimidyl ester (Biotin-XX-SSE) was dissolved in DMSO at a concentration of 0.2 mg/mL and then diluted to a final concentration of 0.5 µg/mL in PBS. αT3-1 cells were serum-starved in DMEM for 2 hours and then placed on ice. Cells were washed twice with cold PBS and then incubated on ice for 15 minutes in 1 mL of Biotin-XX-SSE in PBS per 12cm^2 of culture dish surface area. Cells were then washed twice in cold PBS prior to collection.

Cell Fractionations

To assess the specificity of cell-surface biotinylation, αT3-1 cells were grown to 80% confluence in 10 cm^2 dishes, and surface biotinylated as described above. Cells were scraped into PBS and pelleted by centrifugation. Cell pellets were resuspended in 500 µL of homogenization buffer (HB) containing 250 mM sucrose, 3 mM imidazole, pH 7.4, 2 mM EDTA, and protease inhibitors. A 50 µL aliquot of the whole cell suspension (representing 10% of the total input) was reserved. The remaining suspension was incubated on ice for 10 minutes, homogenized in a glass dounce, and centrifuged for 10 minutes at 3,000 rpm. The post-nuclear supernatant was collected, and centrifuged at 100,000 x g at 4^oC for 30 minutes. The resulting supernatant and pellet (representing cytosolic and total membrane fractions, respectively), as well as the input were boiled in SDS load buffer.

Detergent-resistant, low-density membrane fractions were prepared essentially as described previously (21). Briefly, αT3-1 cells were grown to 70-80% confluence in 15 cm^2 dishes. Following the treatments indicated, cells (~1.5 x 10^8 per dish) were washed twice in cold PBS, and scraped into PBS with protease inhibitors. Cells were pelleted by centrifugation and then resuspended in MES buffer (MBS) containing 25 mM MES, pH6.5, 130 mM NaCl, and protease inhibitors to a final volume of 400 µL. The samples were adjusted to the indicated concentration of Triton X-100 and incubated on ice for 10 minutes. Following dounce homogenization (20 strokes), the samples were mixed with an equal volume of 90% sucrose, placed in a 5 mL ultracentrifuge tube, and overlaid with a discontinuous gradient of sucrose in MBS consisting of 35% (3.7 mL) and 5% (500 µL) layers. The gradients were centrifuged at 116,000 x g in a SW50.1 rotor for 20 hours at 4^oC. Low-density detergent resistant membranes were visible as a band of flocculent material at the 35-5% interface. Fractions (500 µL) were collected starting from the top of the gradient.

Detergent-free fractionations were performed as described previously (51) with minor modifications. αT3-1 cells (~4 x 10^8 cells) were washed and scraped into PBS as described above. Cells were pelleted by centrifugation and washed once in 5 mL cold HB. Cell pellets were resuspended in 2.2 mL HB containing 50 µg/mL cycloheximide, and gently homogenized in a glass dounce (8 strokes). Nuclei and unbroken cells were pelleted by centrifugation and 2 mL of the post-nuclear supernatant were collected and mixed with 2.4 mL of HB-sucrose containing 62% sucrose, 3 mM imidazole, pH 7.4, 2 mM EDTA, and protease inhibitors to achieve a final sucrose concentration in the sample of 40.6%. The homogenate was transferred to a 13 mL ultracentrifuge tube, and overlaid with a discontinuous gradient of HB-sucrose consisting of 35% (4 mL), 25% (3 mL), and HB (600 µL). Samples were centrifuged in a SW41 rotor at 35,000 rpm, 4°C for one hour. Translucent bands of material visible at each interface were collected, diluted four-fold in cold PBS, and repelleted by centrifugation at 100,000 x g for 30 minutes at 4°C. Pellets were resuspended in SDS load buffer and boiled.

Female B6/129 mice (8-20 weeks of age) were euthanized by CO_2 asphyxiation. Pituitaries were collected and placed in DMEM containing 10% fetal bovine serum on ice. Pituitaries were washed once in cold PBS and once in cold MBS, and homogenized as described above. Animal use and experimental protocols for these studies were approved by the Cornell University Institutional Animal Care and Use Committee. For whole pituitary fractionations, pituitaries (n=20) were resuspended in 400 µL cold MBS containing 0.1% Triton X-100, homogenized in a glass dounce (20 strokes), and subjected to discontinuous sucrose density centrifugation as described above for preparation of detergent-resistant low-density membranes.

Preparation of Cell Lysates, Immunoblotting, and Streptavidin Overlay Assays

After the indicated treatments, cells were washed twice in cold PBS, and scraped into cold PBS containing 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine. Cells were pelleted by centrifugation and pellets were resuspended in a radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine. Lysates were cleared by centrifugation and protein concentrations of the lysates were determined by Bradford assay. Protein samples were boiled for 5 minutes in SDS load buffer, resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk (NFDM) in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. For streptavidin overlay assays, blots were blocked as described, and then incubated at room temperature for 1 hour in a 1:10,000 dilution of streptavidin-HRP in TBST. Protein bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer, Boston, MA).

Cholesterol Depletion and Repletion

αT3-1 cells were cultured in 6 cm^2 dishes to 70-80% confluence. Following serum starvation for 2 hours, cells were incubated in DMEM or DMEM containing 2% methyl-β-cyclodextrin (CD) for the indicated times. For cholesterol repletion, CD-containing media was exchanged for DMEM containing 0.5 mg/mL cholesterol in the form of cholesterol-loaded CD for the indicated times. Total cellular cholesterol was measured from 2 uL aliquots of clarified RIPA lysate using a commercial kit

(Amplex Red, Molecular Probes/Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Immunoprecipitation

Aliquots (400 µL) of fractions containing suspensions of low-density membranes, or sucrose alone, were diluted in an equal volume of PBS and adjusted to 0.01% Triton X-100. Anti-GnRHR immune serum (IS, 25 µL), non-immune rabbit serum (NRS, 25 µL), or vehicle (PBS, 25 µL), were added, and the samples were rocked for 4 hours at 4^{o}C. Protein A/G agarose beads (30 µL) were added and the samples were rocked an additional hour at 4^{o}C. Beads were then washed 3 times in PBS with 0.01% Triton X-100, resuspended in 60 µL of SDS load buffer, and boiled.

Statistical Analysis

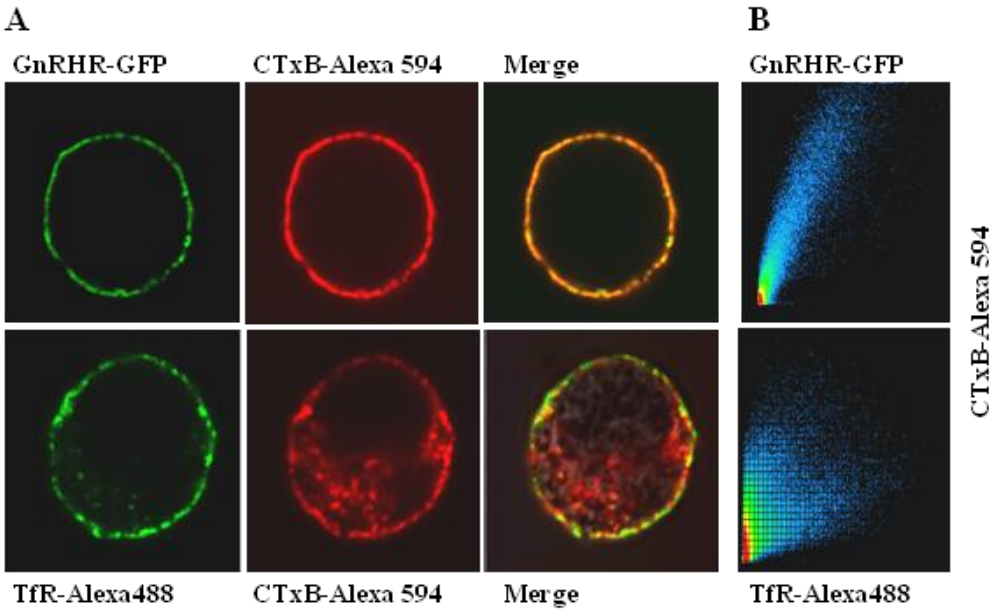
Data on cholesterol concentration were analyzed using a One-way Analysis of Variance with a Bonferoni post-hoc multiple comparisons test. Significance for each pairwise comparison was determined at $p \leq 0.01$.

RESULTS

GnRHR colocalizes with the ganglioside GM_1 in αT3-1 cell plasma membrane.

Low-density membrane microdomains (membrane rafts) are enriched in the ganglioside GM_1. The B-subunit of cholera toxin (CtB) binds specifically to plasma membrane GM_1 and has been used as a marker for GM_1-enriched plasma-membrane subdomains in living cells (22). We have reported biochemical data indicating that the GnRHR resides constitutively within low-density membrane domains in a cholesterol-dependent manner (21). To further characterize the tendency of the GnRHR to

Figure 2.1. GnRHR co-localizes with GM_1 positive membrane domains in αT3-1 cells. A, αT3-1 cells transiently expressing a GnRHR-GFP fusion protein were stained with Alexa 594 conjugated CTxB and fixed. Confocal images of the separated fluorescence channels show membrane localization of the GnRHR-GFP (green channel) and Alexa 594 GM_1 (red channel). Merged images show the overlay of the two flurophores. αT3-1 cells were stained for GM_1 using Alexa 594 CTxB. Cells were then fixed and immunostained for transferrin receptor using an Alexa 488 conjugated second antibody. Confocal images of the separated fluorescence channels show membrane localization of the TfR (green channel) and Alexa 594 GM_1 (red channel). Merged images show the overlay images of the two flurophores. B, Using quantitative colocalization analysis, the merged GnRHR-GFP and Alexa 594 GM_1 image was computed and expressed as a scatter diagram. Co-localization of fluorophores produces a clean diagonal line running from the bottom left to the top right indicating that GnRHR and GM_1 are colocalized on the plasma membrane. In contrast, differences between fluorescence localization causes an irregular distribution in the scatter diagram indicating that TfR and GM_1 display little colocalization.

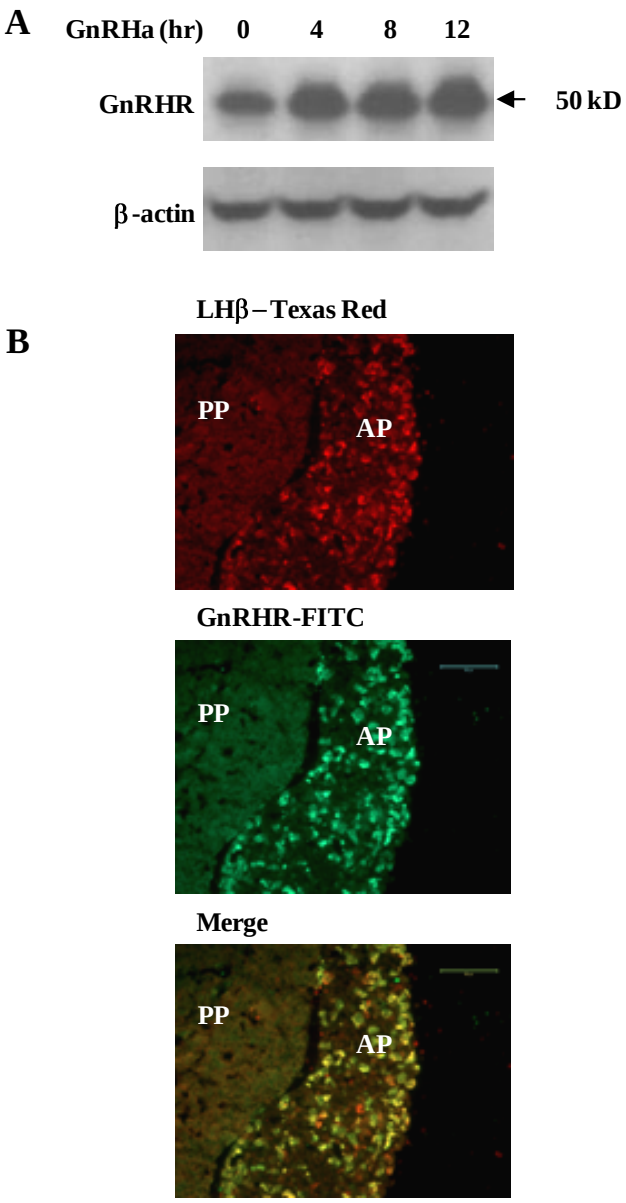

A
B
GnRHR-GFP
CTxB-Alexa 594
Merge
GnRHR-GFP
CTxB-Alexa 594
TfR-Alexa488
CTxB-Alexa 594
Merge
TfR-Alexa488

partition into subdomains of the plasma membrane, we probed αT3-1 cells that stably express a GnRHR-GFP fusion protein with Alexa-594-conjugated CtB and examined the cells by laser confocal scanning microscopy (Figure 2.1). GnRHR-GFP localized exclusively to the plasma membrane and co-localized with GM_1-positive domains. As a control, endogenously expressed transferrin receptor did not co-localize with CtB-Alexa-594/GM_1 positive membrane domains suggesting that GnRHR co-localization with GM_1 was specific in this assay. Consistent with our previous biochemical studies these data indicate that the GnRHR resides constitutively within regions of the plasma membrane marked by GM_1.

Anti-GnRHR immune serum binds specifically to its cognate epitope in both immunoblotting and immunohistochemical applications.

The anti-GnRHR antibody used in these studies was raised against a conserved region of the second extracellular loop of the mammalian type I GnRHR. To validate the performance of this antibody in the studies reported here, we treated αT3-1 cells with GnRHa (the GnRH agonist buserelin) for various time periods and examined whole cell lysates for GnRHR protein expression by immunoblot analysis. As shown in Figure 2.2A, the GnRHR antiserum generated a predominant band of immunoreactivity of approximately 50 kD. Consistent with previous reports of GnRH-induced upregulation of GnRHR in gonadotropes (23), the target of this antibody showed clear upregulation in response to GnRHa. When used for immunohistochemical examination of sections of mouse pituitary, the same antiserum discretely marked a subpopulation of cells within the anterior lobe; co-staining with an antibody against LHβ subunit indicated nearly complete colocalization of GnRHR and LHβ immuno-fluorescence (Figure 2.2B). These validation studies provide evidence

Figure 2.2. Validation of GnRHR antiserum. A, αT3-1 cells were treated with GnRHa for the indicated times. Whole cell lysates were analyzed by SDS-PAGE and immunoblotting using anti-GnRHR immune serum. β-actin was used as a loading control. B, Histological sections of mouse pituitary were probed simultaneously with anti-GnRHR immune serum and an antibody against LH. Sections were then incubated in fluorochrome-conjugated secondary antibodies, mounted, and examined by fluorescence microscopy. PP = posterior pituitary; AP = anterior pituitary.

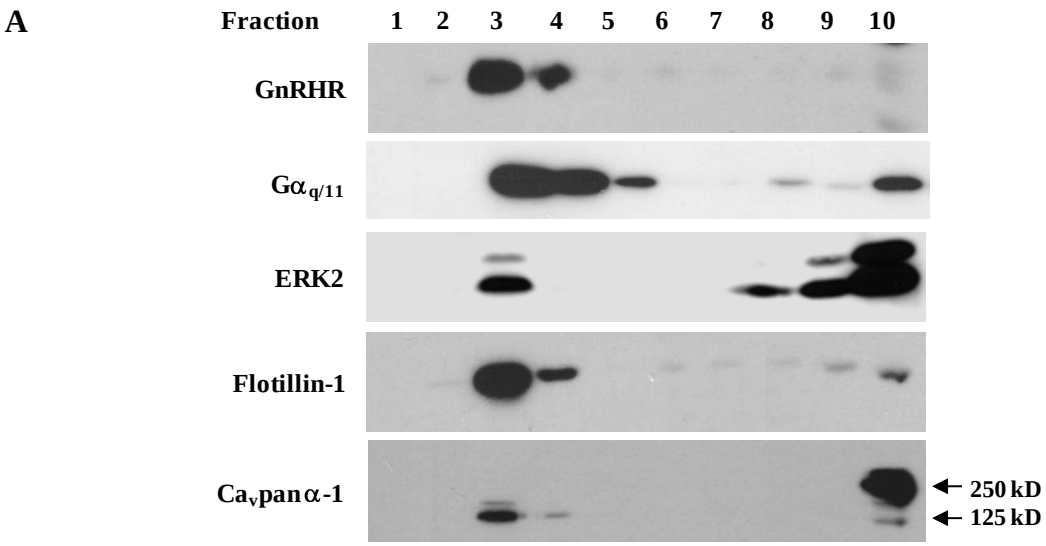

A
GnRHa (hr) 0 4 8 12
GnRHR
50 kD
β-actin
B
LHβ – Texas Red
PP
AP
GnRHR-FITC
PP
AP
Merge
PP
AP

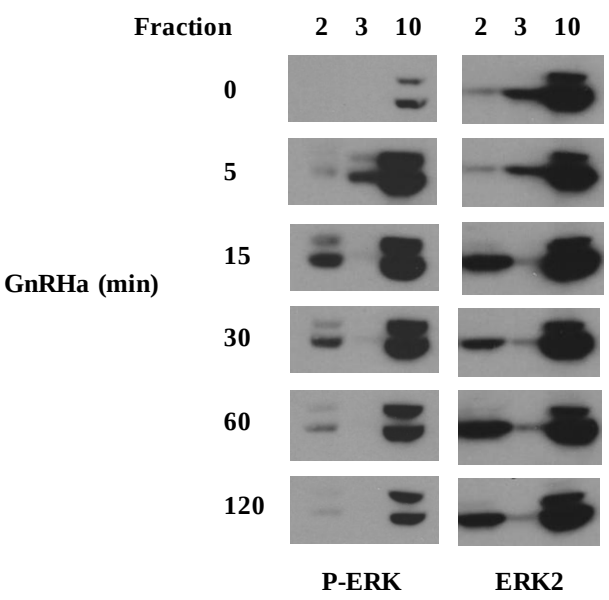
that this antibody recognizes both denatured and non-denatured conformations of its cognate epitope on the GnRHR with high specificity.

ERK 2 associates constitutively with detergent-resistant, low-density membrane domains in αT3-1 cells.

We previously reported that the GnRHR, $G_{\alpha q/11}$, and c-raf, the putative upstream activator of the ERK signaling pathway in gonadotropes, associate constitutively with low-density, detergent-resistant membrane domains (DRMs) in αT3-1 cells (21). Based on this data, we proposed that raft domains may play a key role in GnRH-induced ERK activation by serving as a platform for the association of key intermediates of this signaling pathway. To further explore this model, we fractionated αT3-1 cells in the presence of 0.1% Triton X-100 and analyzed low-density fractions for the presence of the ERK proteins. A small proportion of total cellular ERK 2 partitioned constitutively into low-density fractions (Figure 2.3A). When the microsomal suspensions in the low-density fractions were re-homogenized and repelleted by high-speed centrifugation, ERK immunoreactivity appeared only in the membrane pellet, indicating that the ERK protein recovered in association with DRMs is indeed membrane-associated (data not shown). Phosphorylated ERK was detected in the low-density fractions within 5 minutes of GnRHa administration, and the kinetics of activation of membrane-associated ERK appeared to parallel the kinetics of non-membrane-associated ERK. (Figure 2.3B). Consistent with our previous results, the GnRHR as well as the $G_{\alpha q/11}$ heterotrimeric G-protein subunit and the lipid raft marker flotillin-1 associated preferentially with the low-density fractions (Figure 2.3A). As a further control, the membrane-associated α1 subunit of the L-type voltage gated calcium channel was not present in low-density fractions. This latter

Figure 2.3 ERK2 associates with detergent-resistant, low-density membrane fractions in αT3-1 cells. A, αT3-1 cells were homogenized in the presence of 0.1% Triton X-100 and subjected to discontinuous sucrose density gradient centrifugation. Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting using antibodies against the GnRHR, $G_{\alpha q/11}$, and ERK2, as well as the lipid raft marker flotillin-1, and the non-raft associated Ca_vpan α-1 calcium channel subunit. B, Cells were treated with GnRHa or vehicle for the indicated times and fractionated as in A. Fractions were collected and aliquots of low- and high-density fractions were analyzed by SDS-PAGE and immunoblotting using antibodies against phosphorylated ERK (p-ERK) and ERK2.


A
Fraction
1 2 3 4 5 6 7 8 9 10
GnRHR
Gαq/11
ERK2
Flotillin-1
Cavpanα-1
250 kD
125 kD


B
Fraction
2 3 10 2 3 10
0
5
15
30
60
120
GnRHa (min)
P-ERK
ERK2

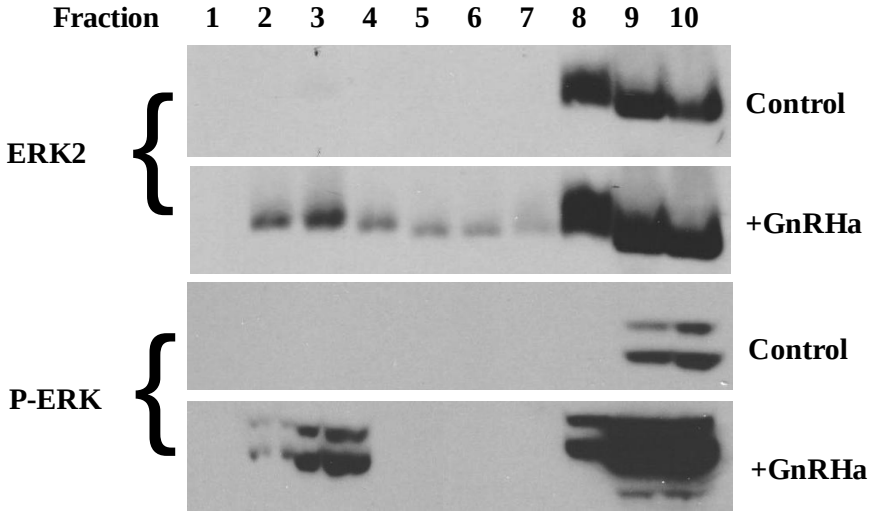


Figure 2.4. GnRHa stabilizes the association of ERK2 with detergent-resistant, low density membrane domains in αT3-1 cells. Cells were treated with GnRHa or vehicle for 15 minutes and then homogenized in the presence of 1.0% Triton X-100. Homogenates were fractionated by discontinuous sucrose density gradient centrifugation and fractions were collected from the top of the gradient. Fractions were analyzed by immunoblot using an antibody against ERK2 (A) or phosphorylated ERK (p-ERK, B).

observation supports the specificity of the fractionation method, and provides a useful negative control for analysis of membrane rafts in gonadotropes.

The DRMs analyzed in Figure 2.3 were isolated following homogenization of cells in the presence of 0.1% Triton X-100. Under these conditions, the association of ERKs with low-density membranes was unaffected by administration of GnRHa (data not shown). In contrast, homogenization of unstimulated cells in the presence of 1.0% Triton X-100 interrupted the association of ERKs with DRMs (Figure 2.4A). However, following 15 minutes of exposure to GnRHa, the association of ERKs with DRMs was rendered refractory to the effects of high detergent concentration (Figure 2.4A), Phosphorylated ERK was also detected in the low-density fractions of GnRHa treated cells following homogenization in the presence of the higher detergent concentration (Figure 2.4B). This observation is consistent with the behavior of c-Raf under similar experimental conditions, and suggests that the signaling activity induced by GnRHR occupancy is capable of stabilizing interactions amongst the participants of a putative raft-associated signaling complex.

ERK2 and the GnRHR associate preferentially with low-density membranes isolated in the absence of detergent.

Resistance to detergent solubilization is an important operational characteristic of membrane rafts (24). Indeed, sucrose density gradient centrifugation of cell homogenates prepared in the presence of low concentrations of non-ionic detergents remains the most common method for isolation of these membrane domains. However, controversy remains regarding the effects of detergents on cellular membranes, and some evidence suggests that detergent solubilization may itself enhance the ability of lipid bilayers to adopt a liquid ordered (l_o) conformation (25).

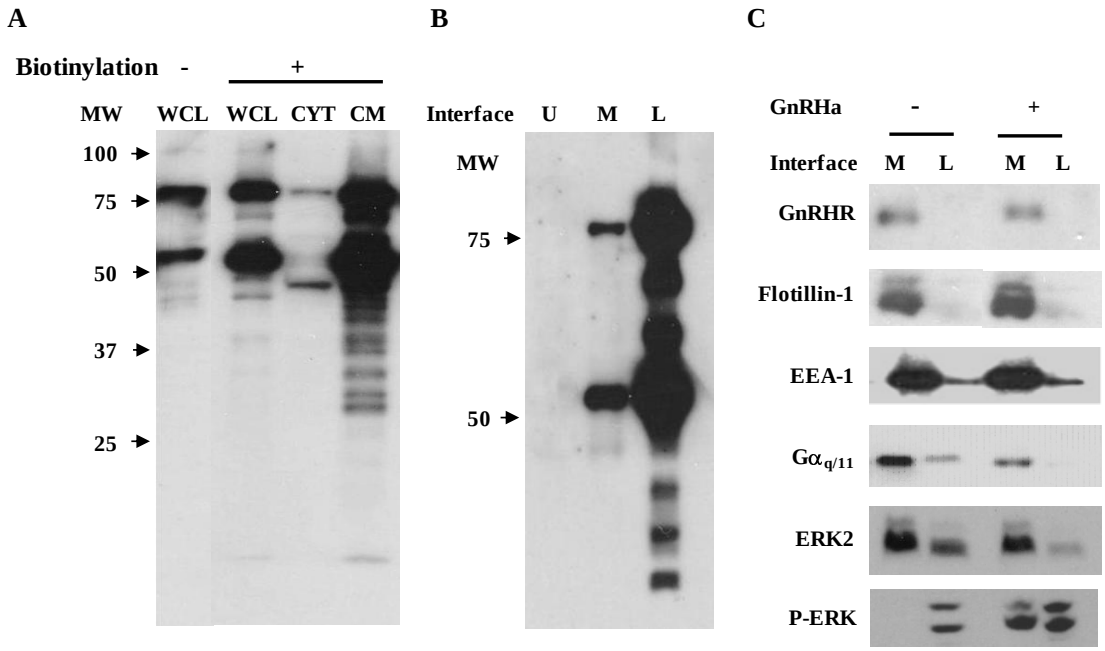


Figure 2.5. In αT3-1 cells, GnRHR and ERK2 associate with low-density membrane domains isolated in the absence of detergent. A, αT3-1 cells were surface biotinylated and separated into whole cell lysate (WCL), cytosolic (CYT), and crude membrane (CM) fractions. Fractions along with non-biotinylated whole cell lysate were subjected to SDS-PAGE and analyzed by streptavidin overlay. Molecular weights are indicated in kD. B,C, cells were homogenized in a detergent-free buffer, and the post-nuclear supernatant was subjected to discontinuous sucrose density gradient centrifugation (40.6, 35, 25, 5%). Microsomal suspensions were collected from the upper (U), middle (M), and lower (L) interfaces of the gradient and the membranes were repelleted by centrifugation at 100,000 x g. Membrane pellets were electrophoresed and analyzed by streptavidin overlay (B) and immuno-blotting using antibodies against the indicated proteins (C).

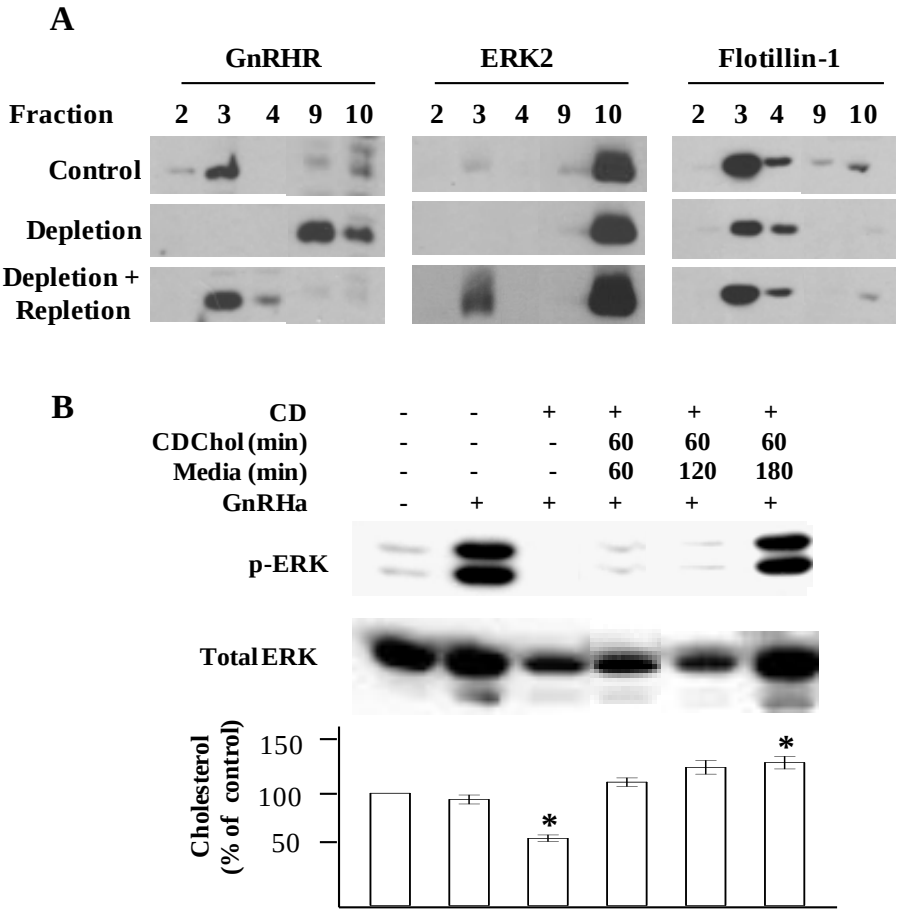
To further assess the validity of our observations regarding the association of ERKs with DRMs, we adapted a technique based on sucrose density gradient centrifugation of post-nuclear supernatant for fractionation of subcellular membranes in the absence of detergent (26). Using this method, Huber and colleagues describe clear separation of low-, intermediate- and high-density subcellular membrane fractions enriched in late endosomes/lysosomes, early endosomes and plasma membrane, respectively. We reasoned that this method might simultaneously result in separation of low- and high-density subdomains of plasma membrane, thereby providing a complementary method for detergent-free isolation of membrane rafts. Initially, we used cell surface biotinylation to track the plasma membrane proteins during the fractionation. Consistent with previous reports (27), in the absence of cell surface biotinylation, streptavidin overlay assays revealed the presence of a small number of endogenously biotinylated proteins in αT3-1 whole cell lysates (Figure 2.5A). Following cell surface biotinylation and separation of cell homogenates into cytosolic and total membrane fractions, streptavidin overlay assays revealed the majority of biotinylation within the membrane fraction, verifying the cell impermeability of the biotinylation reagent, and validating this assay for tracking of bulk plasma membrane during fractionation (Figure 2.5A). Discontinuous sucrose density gradient fractionation of αT3-1 cell postnuclear supernatants following cell surface biotinylation revealed that the majority of plasma membrane localized to the high density fraction (35-40% sucrose interface, Figure 2.5B). Microsomal suspensions retrieved from the middle fraction of the sucrose gradient (25-35% sucrose interface) were enriched in the early endosomal marker EEA-1 (Figure 2.5C). However, both the GnRHR and the lipid raft marker flotillin-1 partitioned exclusively into this middle fraction, indicating that this technique allows effective separation of low-density subdomains of plasma membrane. Both the ERKs as well as $G_{\alpha q/11}$ associated with the lower density middle

fraction in a manner unaffected by GnRHa treatment (Figure 2.5C). Following GnRHa treatment, phosphorylated ERK was similarly detected in the lower density middle fraction. Immunoreactivity for the proteins examined was undetectable in the low-density upper fraction (data not shown).

Both the association of ERK2 with low-density membrane domains as well as ERK inducibility by GnRHa are susceptible to perturbation of cellular cholesterol levels.

Analysis of lipid behavior in model membranes indicates that l_o domain formation is highly dependent upon cholesterol (28). Cholesterol is also understood to be critically important in the formation of lipid raft domains in living cells (29). We previously reported that, in αT3-1 cells, depletion of cellular cholesterol led to a dissociation of the GnRHR from a detergent-resistant, low-density membrane environment as well as a reversible uncoupling of the ability of GnRH to activate the ERK pathway (21). To further define the importance of cholesterol-dependent membrane organization for the function of this signaling pathway, we used a depletion/repletion strategy to define the kinetics of recovery of GnRH-induced ERK activation following cholesterol perturbation. Exposure of cells to methyl-β-cyclodextrin (CD) for 40 minutes resulted in a complete loss of both the GnRHR and the ERKs from DRMs (Figure 2.6A). However, the association of the lipid raft marker flotillin-1 with DRMs was unaffected, indicating that cholesterol depletion did not promote complete solubilization of this membrane compartment (Figure 2.6A). Consistent with our previous results, CD exposure resulted in a significant decrease in cellular cholesterol to approximately 50% of control levels and led to a complete block of ERK activation by GnRH (Figure 2.6B). Repletion of cholesterol by exposure of

Figure 2.6. Normalization of cellular cholesterol restores the association of ERK2 with low-density membrane domains in αT3-1 cells following cholesterol depletion, but this is not sufficient to reconstitute cholesterol-dependent ERK inducibility by GnRH. A, Cells were cultured in the absence (Control) or presence (Depletion) of methyl-β-cyclodextrin (CD) for 40 minutes. Some cells were then incubated in media containing 0.5 mg/mL of cholesterol in the form of cholesterol-loaded CD (CDChol) for 60 minutes (Depletion + repletion). Cells were homogenized in the presence of 0.1% Triton X-100 and fractionated by discontinuous sucrose density gradient centrifugation. Fractions were analyzed by immunoblot using anti-GnRHR immune serum, or antibodies against ERK2 and the lipid raft marker Flotillin-1. B, Cells were subjected to cholesterol depletion and repletion as in (A). Some samples were then incubated for the indicated times in cholesterol-free media (Media). Whole cell lysates were analyzed by immunoblot for phosphorylated and total ERK proteins. Total cellular cholesterol measurements were normalized to total protein concentrations and are expressed as mean +/-SEM for 4 separate experiments. Asterices indicate significant differences from the control group ($p \leq 0.01$)

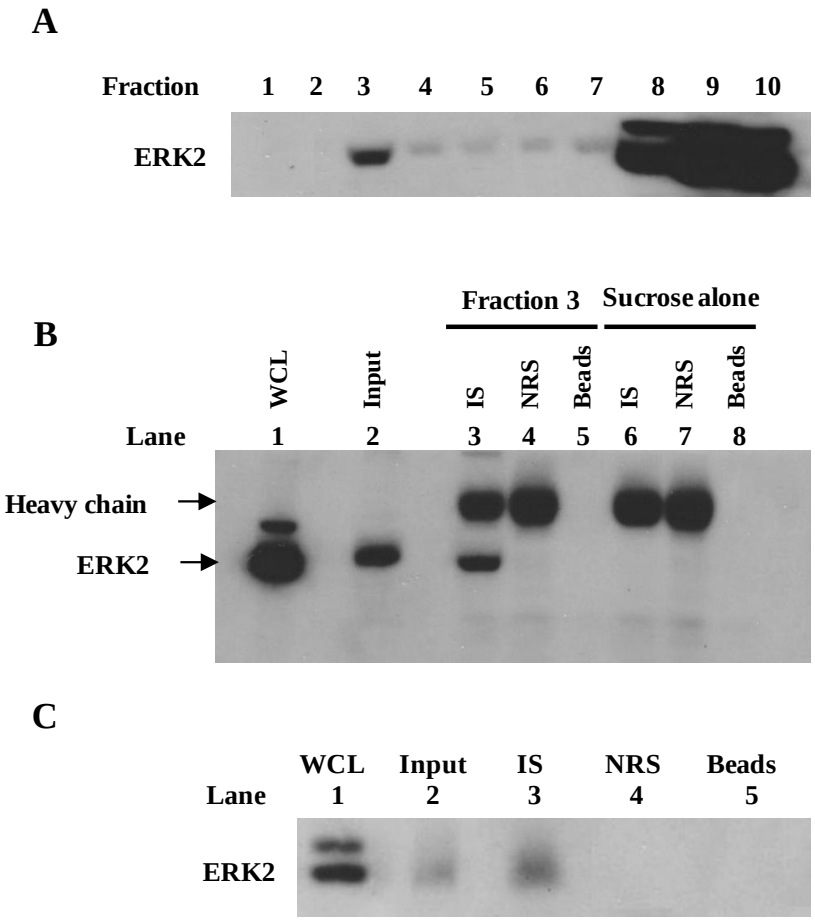

A
GnRHR
ERK2
Flotillin-1
Fraction
2 3 4 9 10
2 3 4 9 10
2 3 4 9 10
Control
Depletion
Depletion + Repletion
B
CD
CDChol (min)
Media (min)
GnRHa
p-ERK
Total ERK
Cholesterol (% of control)
150
100
50
*
*

cells to CD pre-loaded with cholesterol (CDChol) for 60 minutes normalized cellular cholesterol levels and led to reassociation of both the GnRHR and ERKs with DRMs (Figure 2.6A). However, despite normalization or even mild elevation of total cellular cholesterol, ERK inducibility by GnRH was not restored for up three hours (Figure 2.6B). Moreover, exposure of αT3-1 cells to CDChol alone led to an approximately 3-fold increase in cellular cholesterol levels and caused a block in ERK activation by GnRH that developed with highly similar kinetics (data not shown). These results suggest that cholesterol homeostasis is critical for normal signaling through the GnRHR and implicate a complex process of dynamic membrane (re-)organization as an important regulatory feature in the functional re-establishment of the ERK pathway following perturbation of cholesterol homeostasis in cell membranes.

GnRHR and ERK2 co-immunoprecipitate from low-density membrane domains in αT3-1 cells.

Our experimental results to date suggest the possibility that ERK activation following GnRHR occupancy may be initiated through the interaction of several signaling intermediates ($G_{\alpha q/11}$, c-Raf, ERKs) that are compartmentalized in proximity to the GnRHR through association with low-density plasma membrane microdomains. However, our data do not rule out the possibility that co-fractionation of multiple signaling proteins in this pathway represents the isolation of a complex, mixed population of subcellular membrane compartments. For example, many components of the ERK pathway have been shown in other systems to be assembled in association with endosomal membranes through interactions with specific scaffolds and adaptors (30, 31). To further determine whether the GnRHR and ERKs associate with a common membrane environment within the raft compartment, we immunoprecipitated

Figure 2.7. GnRH receptor and ERK2 co-immunoprecipitate from low-density membrane fractions in αT3-1 cells. A, Cells were homogenized in the presence of 0.1% Triton X-100 and fractionated through a discontinuous sucrose gradient. Fractions were collected and aliquots were analyzed by immunoblot using an antibody against ERK2. B, GnRHR was immunoprecipitated from fraction 3 of the experiment in A (lanes 3-5) using anti-GnRHR immune serum (IS), nonimmune serum (NRS) or beads alone (Beads). Control immunoprecipitations were performed in sucrose alone (lanes 6-8). Whole cell lysate (WCL, 1 ug lane 1), 5% of the input for each IP (Input, lane 2) and the immunoprecipitates were analyzed by immunoblot using an anti-ERK2 antibody. C. Cells were homogenized in the absence of detergent and fractionated by discontinuous sucrose density gradient centrifugation (40.6, 35, 25, 8%). GnRHR was immunoprecipitated from microsomal suspensions at the 25-35% sucrose interface using anti-GnRHR immune serum (IS), nonimmune serum (NRS) or beads alone (Beads). Whole cell lysate (WCL, 1 µg, lane 1), 5% of the input for each IP (Input, lane 2) and the immunoprecipitates (lanes 3-5) were analyzed by immunoblot using an anti-ERK2 antibody.


A
Fraction 1 2 3 4 5 6 7 8 9 10
ERK2
B
Fraction 3
Sucrose alone
WCL
Input
IS
NRS
Beads
IS
NRS
Beads
Lane 1 2 3 4 5 6 7 8
Heavy chain
ERK2
C
WCL
Input
IS
NRS
Beads
Lane 1 2 3 4 5
ERK2

the GnRHR from low-density membrane suspensions and examined the immunoprecipitates for the presence of ERKs. ERKs co-immunoprecipitated with the GnRHR from DRMs (Figure 2.7A and B), as well as from microsomal suspensions prepared in the absence of detergent (Figure 2.7C). The reciprocal immunoprecipitations targeting ERK2 followed by immunoblot analysis for the GnRHR were uninformative due to a high level of non-specific signal arising from the heavy chain of the anti-ERK2 primary antibody.

GnRHR associates with low-density membrane domains in whole mouse pituitary, and co-immunoprecipitates with ERK2 in mouse pituitary homogenates.

Our data from αT3-1 cells strongly implicate low-density membrane microdomains in the regulation of ERK activation by GnRH. To determine whether these domains may play a similar role in organization of this signaling pathway in differentiated gonadotropes in vivo, we examined DRMs from homogenates of whole mouse pituitaries. The GnRHR partitioned into low-density DRMs in a manner similar to that observed in αT3-1 cells (Figure 2.8A). Flotillin-1 was present exclusively in the low-density fractions, while transferrin receptor was detected only in the high-density solubilized fractions (data not shown). ERKs were also detectable in low density fractions from pituitary homogenates (Figure 2.8A). Whole pituitary represents a diversity of cell types in which ERKs are ubiquitously expressed; thus the presence of ERKs within low-density fractions of pituitary homogenates does not provide evidence for cell type specificity regarding the association of ERKs with low-density membranes. To further determine whether ERKs associate with a low-density membrane compartment in gonadotropes, we immunoprecipitated the GnRHR from

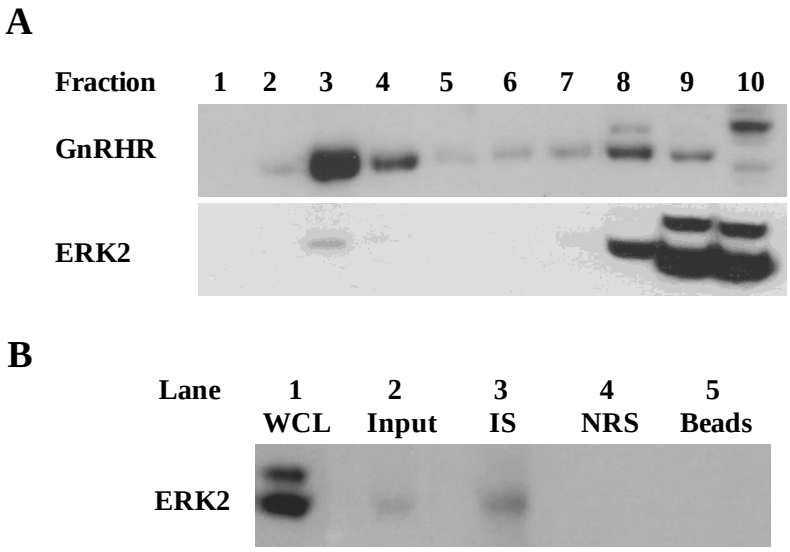


Figure 2.8. GnRHR and ERK2 co-immunoprecipitate from detergent-resistant, low-density membranes in whole mouse pituitaries. A, whole mouse pituitaries were homogenized in the presence of 0.1% Triton X-100 and subjected to discontinuous sucrose density gradient centrifugation. Fractions were collected from the top of the gradient and aliquots of each fraction were analyzed by immunoblot using anti-GnRHR immune serum and anti-ERK2 antibody. B, GnRHR was immunoprecipitated from fraction 3 of the same experiment using anti-GnRHR immune serum (IS), nonimmune serum (NRS) or beads alone (Beads). Whole cell lysate (WCL, 1 ug, lane 1), 5% of the input for each IP (Input, lane 2) and the immunoprecipitates (lanes 3-5) were analyzed by immunoblot using an anti-ERK2 antibody.

DRMs of pituitary homogenates and examined the precipitates for the presence of ERKs. Consistent with our observations in αT3-1 cells, ERKs co-immunprecipitated with the GnRHR indicating that these proteins associate with a common membrane environment in vivo (Figure 2.8B).

DISCUSSION

Subcellular compartmentalization of signaling proteins is now recognized as a mechanism by which signaling activity may be temporally and spatially regulated within the cell, or by which the activation of a given signaling pathway may be linked to a diversity of biological outcomes (32-34). This has become particularly evident in the area of GPCR signaling. While early studies of GPCR function focused on the role of activated G-protein α subunits in the generation of diffusible second messengers and modulation of ion conductances, current models emphasize the spatial segregation and trafficking of cell surface receptors amongst discrete subdomains of the plasma membrane, as well as the ability of internalized GPCRs to couple to diverse intracellular signaling cascades, including the ERK module of the MAPK system (35). Consistent with these themes, we previously demonstrated that, unlike other GPCRs, the GnRHR partitions constitutively and exclusively into membrane rafts, and that this raft association was required for the ability of this GPCR to couple to the ERK pathway in the αT3-1 gonadotrope cell line (21). The results described here expand upon these earlier findings and show that the GnRHR partitions into low-density membrane domains in whole mouse pituitary as well in αT3-1 cells. These results further support the use of this cell line as a model of GnRHR behavior in pituitary gonadotropes, and suggest that compartmentalization of the GnRHR into low-density membrane microdomains is of functional significance in vivo.

Following ligand activation, most GPCRs are rapidly phosphorylated, desensitized, and targeted for clathrin-mediated endocytosis through association with β-arrestins. (36). Arrestins have also been shown to provide an important link between many GPCRs and the ERK pathway by nucleating specific signaling complexes on the surface of receptor-laden early endosomes (37-39). However, the GnRHR is not phosphorylated following ligand binding, and does not associate with arrestins (40). Furthermore, the delayed kinetics of GnRHR internalization suggests that GnRH-induced ERK activation occurs independently of receptor internalization (41). Previously, we proposed a model in which coupling of the GnRHR to the ERK pathway involves, at least in part, the assembly of a raft-associated signaling complex consisting minimally of the GnRHR, $G_{\alpha q}$, and c-Raf (21). Our present confocal imaging data shows that the vast majority of the GnRHR population localizes to GM1-enriched regions of the plasma membrane in αT3-1 cells. Furthermore, we show that the GnRHR and ERKs co-immunoprecipitate from low-density membrane fractions both in αT3-1 cells as well as whole mouse pituitary. These data demonstrate a close physical proximity between the GnRHR and ERKs at the level of the plasma membrane and provide further support for our model of a specific plasma membrane-associated signaling complex as a link between the GnRHR and the ERK pathway in gonadotropes.

In these studies, the behavior of the ERKs with respect to their association with low-density membrane domains was similar to our previous observations of c-Raf (21). Both c-Raf and the ERKs partitioned constitutively and partially into low-density membrane fractions, both in αT3-1 cells and in whole mouse pituitary. In addition, these kinases were rapidly activated within this membrane compartment following GnRHa stimulation (21). The small proportion of total cellular ERKs that partitioned into the low-density membrane compartment may reflect the overall small

size of this compartment, or may indicate a relative paucity of ERK docking sites within this compartment, dictated perhaps by the specific architecture of raft-associated complexes. Also, since signaling complexes are characterized by low-affinity interactions, it is possible that dissociation and loss of some of these complexes from low-density membranes during homogenization and fractionation led to an underestimation of the proportion of ERKs that reside within this compartment in intact cells. Like c-Raf, the association of ERKs with membrane rafts in resting cells was completely disrupted by homogenization of cells in higher concentrations of the non-ionic detergent Triton X-100. Importantly, however, stimulation with GnRHa rescued the raft-association of ERKs, despite exposure of cells to higher detergent concentrations. The biophysical basis for this increased resistance to detergent solubilization is unclear; however, the apparent increase in affinity of both c-Raf and ERKs for the low-density membrane compartment that is induced by GnRH argues that modulation of this membrane affinity is a specific biological effect of GnRHR activation. Finally, unlike c-Raf, association of ERKs with low-density membranes was susceptible to cholesterol depletion, and was restored following cholesterol replenishment, in a manner that paralleled the GnRHR. Interestingly, ERKs do not possess a defined lipid binding domain, and, to our knowledge, have not been shown to interact directly with membranes (42). Loss of ERKs from the low-density membrane compartment under conditions of cholesterol depletion likely reflects the cholesterol dependence of an intermediate, membrane-associated adapter protein(s). Our analysis of the kinetics of recovery of ERK inducibility following cholesterol depletion and repletion indicated that normalization of total cellular cholesterol levels and restoration of the raft-association of the GnRHR and ERKs are not sufficient for reconstitution of signaling. This temporal lag in recovery of signaling is consistent

with a requirement for reorganization of a raft-associated multiprotein signaling complex.

While numerous methods for the study of membrane rafts have been reported, the most commonly used method for their isolation remains extraction of cells with low concentrations of a nonionic detergent, followed by fractionation of the cell homogenate by density gradient centrifugation. However, studies of model membranes have raised concerns regarding the ability of non-ionic detergents to promote the formation of a cholesterol-dependent l_o phase (24, 43). To address these concerns, our previous work analyzed both DRMs as well as membrane fractions prepared using a detergent-free, carbonate-based method to demonstrate association of the GnRHR with low-density membranes (21). Here we report the use of an additional complementary technique for fractionation of subcellular membranes in the absence of detergent. This method was originally used for isolation of microsomal suspensions enriched in early endosomes (26). Huber and colleagues have shown that early endosomes are largely separable from plasma membrane on the basis of differential buoyant density alone (30). Since membrane rafts are distinguishable from bulk plasma membrane by their lower buoyant density, as well as their detergent-resistance, we reasoned that this technique might allow separation of membrane rafts from bulk plasma membrane in the absence of detergent. Indeed, our results with this method show that both the GnRHR and the lipid raft marker flotillin-1 partitioned into a low-density microsomal fraction that is enriched in early endosomes. Using cell-surface biotinylation to track the plasma membrane during the fractionation, our data indicate that the majority of plasma membrane partitions into a higher density fraction, consistent with the notion that the membrane raft domains occupied by GnRHR constitute a small proportion of the total plasma membrane in these cells. Interestingly, membrane-associated ERKs showed a preference for the lower density

fraction. Since this low-density fraction represents a mixed population of endosomal and plasma membrane elements, we cannot exclude the possibility that a significant proportion of the ERKs that partitioned into this fraction were associated with endosomes. Indeed assembly of signaling complexes on endosomes is a prominent feature of the organization of the ERK pathway (30, 44). However, our ability to co-immunoprecipitate the GnRHR and ERKs from this fraction indicates that at least some of the ERKs within this compartment are associated specifically with GnRHR-enriched plasma membrane rafts. The appearance of phosphorylated ERK within this fraction following GnRHa treatment further supports this conclusion. Overall, these data corroborate our analyses of DRMs and lend further support to our model of a raft-based GnRHR signaling platform.

While our data provide evidence for the association of ERKs with plasma membrane rafts in gonadotropes, the functions and substrates of this membrane-associated ERK are unknown. In most cell types, including gonadotropes, a substantial portion of activated ERK undergoes nuclear translocation following activation; indeed, phosphorylation of nuclear substrates involved in transcriptional regulation is often considered the predominant endpoint of pathway activation. However, ERKs have also been shown to target a variety of extranuclear substrates including cytoskeletal regulatory proteins and cytoskeletal elements, proteins involved in intracellular membrane trafficking, proteosomal subunits, and translational regulators (45-47). While the functional link between the ERK pathway and the activity of many of these substrates has not been thoroughly defined, a recent report indicated that the ERKs play a key role in the GnRH-induced upregulation of LHβ mRNA translation in LβT2 cells through phosphorylation of specific elongation initiation factors (48). As a further example of the complex and multifunctional organization of this pathway, it has been shown that mitogen-induced activation and

nuclear translocation of the ERK substrate p90RSK1 is preceded by its transient translocation to the plasma membrane via a mechanism that involves ERK interaction, but that is independent of ERK phosphotransferase activity (49). This raises the interesting possibility that, in addition to their prominent role as kinases, activated ERK proteins may serve adapter roles under certain circumstances. Given the small proportion of total cellular ERK that resides within the raft compartment in our studies, it seems unlikely that the totality of ERK activation that occurs within these cells takes place within the context of the raft-associated GnRHR signaling platform proposed in our model. However, we are intrigued by the possibility that raft-associated ERKs play a role in the cellular response to GnRH by targeting a specific subcellular complement of substrates. Determination of the functional importance of raft-associated ERKs in gonadotropes will require identification of the molecular determinants required for association of these proteins with membrane rafts, as well as the composition of the protein complexes that form within this compartment.

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CHAPTER 3

ERK SIGNALING IN THE PITUITARY IS REQUIRED FOR FEMALE BUT NOT MALE FERTILITY

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SUMMARY

Males and females require different patterns of pituitary gonadotropin secretion for fertility. The mechanisms underlying these gender-specific profiles of pituitary hormone production are not known; however, they are fundamental to understanding the sexually dimorphic control of reproductive function at the molecular level. Several lines of evidence have suggested that the extracellular signal-regulated kinases ERK1 and 2 are essential modulators of hypothalamic GnRH mediated regulation of pituitary gonadotropin production and fertility. To test this hypothesis, we generated mice with a pituitary specific depletion of ERK1 and 2 and examined a range of physiological parameters including fertility. We find that ERK signaling is required in females for ovulation and fertility, while male reproductive function is unaffected by this deficiency. The effects of ERK pathway ablation on luteinizing hormone production underlie this gender specific phenotype, and the molecular mechanism involves a requirement for ERK-dependent upregulation of the transcription factor Egr1, which is necessary for LHβ expression. Together, these findings represent a significant advance in elucidating the molecular basis of gender specific regulation of the hypothalamic-pituitary-gonadal axis and sexually dimorphic control of fertility.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) signaling pathways comprise a conserved set of signal transduction modules that are activated in response to a variety of extracellular stimuli (1). The extracellular signal-regulated kinase (ERK) pathway is the most thoroughly characterized of the MAPK systems and consists of a 3-level phosphorylation cascade which, in its canonical form, includes the MAPK-kinase-kinase Raf-1, the MAPK-kinases MEK1 and 2, and the MAPK's ERK1 (MAPK3) and ERK2 (MAPK1). Activated ERKs phosphorylate a multitude of targets throughout the cell, exerting broad regulatory influence over a wide range of processes including transcription, translation, cell cycle regulation, cytoskeletal remodeling, and apoptosis (1).

ERK1 and 2 are generally thought to serve overlapping functions; however, it has also been demonstrated that ERK1 and 2 may play quite distinct roles in some differentiated cells (2). In addition, the ERK1 null mouse is viable and fertile while the ERK2 null is embryonic lethal (3, 4). Thus, ERK1 and 2 clearly serve highly divergent functions during development (5). A genetic model that would allow for systematic analysis of the functions of ERK1 and 2 within specific cellular or developmental contexts is highly desirable; however, the embryonic lethality of the ERK2 null mouse necessitates a conditional approach to the ablation of ERK2, and has presumably hampered development of such a model.

The pituitary gland is a complex endocrine organ that regulates many aspects of mammalian homeostasis. The anterior pituitary is composed of several major hormone-producing cell types including thyrotropes that produce thyrotropin-stimulating hormone (TSH); and gonadotropes that produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH). TSH, LH, and FSH are heterodimeric hormones that have distinct β subunits but share a common α subunit (αGSU). The

αGSU is the first differentiated cell marker to appear during pituitary development (6). Reporter assays suggest that the *αGSU* promoter may become activated as early as e9.5 throughout the pituitary primordium; however, subsequent expression of αGSU becomes restricted to thyrotropes and gonadotropes (7-9).

Thyroptropes and gonadotropes play important roles in the regulation of metabolic and reproductive function, respectively. Stimulation of these cells by the hypothalamic releasing hormones thyrotropin-releasing hormone (TRH) or gonadotropin-releasing hormone (GnRH) leads to synthesis and secretion of TSH by thyrotropes, and FSH and LH by gonadotropes. The ERK pathway is activated in both thyrotropes and gonadotropes following stimulation by TRH or GnRH. Little is known regarding the importance of the ERK pathway in thyrotropes. In contrast, many studies suggest that ERK signaling is critical for the expression of several genes essential for the function of gonadotropes, including the *αGSU, LHβ*, and a regulatory MAP kinase phosphatase (*MKP2/DUSP4*) (10-12). Data supporting the importance of ERK signaling for gonadotrope function have been generated primarily through study of the αT3-1 and LβT2 gonadotrope-derived cell lines. These cell lines have been useful tools in the molecular dissection of gonadotrope function; however, they are isolated from the complex endocrine environment of the living animal. In light of the need for an in vivo model for study of the role of ERK signaling in the pituitary, we used Cre-LoxP technology to generate mice in which ablation of ERK1 and 2 were targeted to the αGSU-expressing cells of the anterior pituitary. Our results demonstrate that ERK signaling is required for fertility only in the female and provide new insight into the mechanisms underlying sexually dimorphic regulation of reproductive function.

MATERIALS AND METHODS

Animals

ERK1 null (*ERK1*$^{-/-}$) and ERK2 floxed (*ERK2*$^{fl/fl}$) mice have been described previously (33, 34). *αGSU:CRE* mice were purchased from Jackson Laboratories (Bar Harbor, ME). Swiss Webster mice used as wild type controls were obtained from Taconic Farms (Germantown, NY). Rosa 26 reporter (R26R) mice were generously provided by Dr. Michael Kotlikoff (Cornell University, Ithaca NY). Animals were handled in compliance with the Cornell University Institutional Animal Care and Use Committee. For assessment of fertility, females were individually housed with males of proven fertility and examined daily for vaginal plugs. Upon identification of a plug, females were isolated and observed for pregnancy. Females in which vaginal plugs were not identified, or that failed to develop pregnancy after identification of a plug, were reintroduced to new males, for a total of 21 days of breeding opportunity. Following the breeding challenge, females were isolated for 14 days and then sacrificed and examined for pregnancy post mortem. For superovulation, mice were injected intraperitoneally with either 5 IU pregnant mare serum gonadotropin (PMSG) or vehicle, followed in 48 hours by injection of 5 IU of either human chorionic gonadotropin (hCG) or vehicle. After 72 hours, mice were sacrificed and ovaries were collected for histological examination. Ovariectomy and castration were performed via ventral midline celiotomy under isoflurane anesthesia using standard aseptic conditions.

Genotyping

Genomic DNA was isolated from tail snips (3 mm), or an equivalent quantity of other tissues as indicated, using a commercial kit (Qiagen DNeasy, Valencia, CA) as per the manufacturer's instructions. Routine genotyping of animals was performed

by PCR as previously described (5). PCR for confirmation of recombination at the *ERK2* locus was performed using primers 'F': 5'-AGCCAACAATCCCAAACCTG-3', '5': 5'-GCTGCCTAGAAACATGGAGCTGC-3', '7': 5-GCTCTTTAACCTCCACTGCCTAAGC-3', and '9': 5'-GCCAGCTGCTCACACTTAGCAAAGC-3'. The identities of PCR products were confirmed by direct nucleotide sequencing for all founder animals.

Histology, Immunohistochemistry, and Immunofluorescent labeling

For histological examination, tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4μm, stained with hematoxylin and eosin (H&E) using standard histological technique, and examined by light microscopy. In some studies, ovaries were serially sectioned and every 4^{th} section was examined microscopically for identification of luteal tissue.

For ERK2 immunohistochemistry, sections were deparaffinized in xylene, rehydrated through EtOH dilution series to distilled H_20. Antigen retrieval was performed by boiling the slides in 0.01M Citrate buffer (pH 6.0). Sections were washed in PBS, and blocked with 10% normal rabbit serum/10% nonfat dry milk in PBS for 20 minutes at room temperature. Sections were then incubated in goat anti-ERK2 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:60 in PBS/1X casein (Vector Laboratories, Burlingame, CA), for two hours at 37^oC. Sections were further washed in PBS, and incubated at room temperature for 20 minutes with biotinylated rabbit-anti-goat IgG (Invitrogen, Carlsbad, CA). Sections were then treated with ABC reagent and diamino-benzidine as per the manufacturer's recommendations (Vector).

For immunofluorescent co-labeling of ERK2 and LHβ, 5 μM pituitary sections were deparaffinized and rehydrated as described above. Antigen retrieval and

incubation in anti-ERK2 primary antibody were also performed as above, substituting normal goat IgG at equivalent concentration (ug/mL) as negative control. After incubation with biotinylated rabbit-anti-goat IgG and further washing, sections were incubated with Streptavidin Alexa Fluor® 488 (Invitrogen) for 20 minutes at room temperature in the dark. Stained slides were washed further with PBS and stored in distilled water overnight at 4^{o}C. On day two, sections were blocked with Fab Fragment goat anti-rabbit IgG H&L (Jackson ImmunoResearch, West Grove, PA) diluted 1:50 in PBS for 30 minutes at 37^{o}C, and reblocked for 20 minutes with 10% goat serum/2Xcasein (Vector) in PBS. Rabbit anti-LHβ primary antibody (National Hormone and Peptide Program; NIDDK) was reconstituted in PBS at a concentration of 1 μg/μL, and applied at a 1:50 dilution for 2 hours at 37^{o}C, substituting normal rabbit IgG (Vector) at an equivalent concentration (ug/mL) as a negative control. LHβ was detected with Texas Red goat-anti-rabbit IgG H&L (Vector) at a 1:80 dilution in PBS for 20 minutes at room temperature in the dark. Slides were washed and mounted in Vectashield DAPI (Vector). Images were obtained on a Nikon E400 epi-fluorescence microscope using the appropriate filters.

Vaginal Cytology

Vaginal lavage was performed with 30 μL of PBS and the effluent was used to make routine cytological smears. Slides were fixed in methanol, and stained with Wright's stain. Slides were examined by light microscopy and epithelial cells and leukocytes were differentiated on the basis of cell morphology.

Serum hormone measurements

Immediately following euthanasia, mice were exsanguinated by cardiac puncture. Blood samples were allowed to clot at room temperature for 2 hours and

were then centrifuged at 8,000 rpm for 10 minutes. Serum supernatants were collected and stored at -80^{o}C until assayed. Total serum T4 concentrations were determined by ELISA using a commercial kit according to the manufacturer's instructions (Alpha Diagnostics, San Antonio, Texas) with samples run in duplicate. Measurements of LHβ and FSHβ were performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core facility with samples run in singlet.

Immunoblotting

Tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0.2 mM PMSF, 5 mM benzamidine. Lysates were cleared by centrifugation and protein concentrations were determined by Bradford assay. Protein samples were boiled for 5 minutes in SDS load buffer, resolved by SDS-PAGE, and transferred to PVDF membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk (NFDM) in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then incubated with specified antisera (anti LHβ, FSHβ and TSHβ from the National Hormone and Peptide Program; NIDDK; and anti-actin and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz). Protein bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer, Boston, MA).

Pituitary Cell Primary Culture

Mice were euthanized and pituitaries were placed in DMEM containing 10% FBS on ice. Tissues were digested with agitation at 37^{o}C for 10 minutes in DMEM containing 0.5 mg/mL each of collagenase and hyaluronidase (Sigma Chemical, St

Louis, MO). Following tritiuration, tissue remnants were allowed to settle by gravity. The supernatant was removed and the tissue pellet was resuspended in DMEM containing 0.25 mg/mL each of the same enzymes, and digested for an additional 10 minutes. Following a second round of tritiuration and sedimentation, the supernatants were combined. Cells were washed once in DMEM containing 10% FBS, and plated in like medium on 15 mm diameter poly-L-lysine coated dishes at a density of 2 pituitary equivalents per well. Cells were maintained at 37^{o}C in 5% CO_2 for 12 hours before treatment.

RNA isolation and Quantitative PCR

Following the indicated treatments, total RNA was isolated from cultured cells or whole pituitaries using the RNeasy kit (Qiagen) according the manufacturer's instructions. Reverse transcription was carried out in 40 μL reaction volumes using the High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA). Taqman primer-probe sets for mouse LHβ, αGSU, FSHβ, GnRHR, TSHβ and Egr1, were purchased commercially (Applied Biosystems). Amplification was performed under standard conditions using the ABI Prism 7500 Sequence Detection System. Transcript levels were normalized to corresponding levels of β-actin, and were calibrated to corresponding transcript levels represented in a pooled cDNA stock derived from wild type female mice, or to levels of the control group, as indicated.

Data analysis

Quantitative PCR and ELISA results were analyzed by t-test or one-way ANOVA. Post hoc tests were performed with Bonferroni all-pairwise comparisons. All differences were considered significant at $p \leq 0.05$.

RESULTS

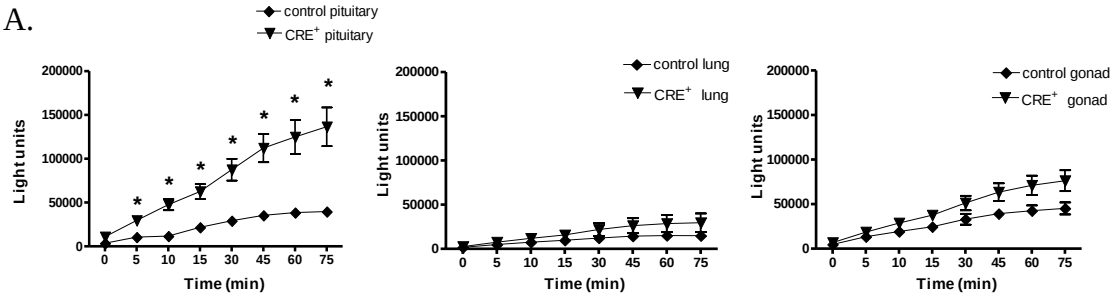
Generation and validation of the pituitary-targeted ERK1/2 double knockout mouse

To define the physiological importance of ERK signaling in pituitary gonadotropes and thyrotropes, we began by generating mice with a conditional, pituitary-targeted ablation of ERK2. Mice homozygous for a floxed mutation at the *ERK2* locus (*ERK2*$^{fl/fl}$), were crossed with αGSU:Cre mice, in which Cre recombinase is expressed under the regulatory control of a 4.6 kb fragment of the murine αGSU promoter. *ERK2*$^{fl/fl}$, *αGSU:Cre* mice were then mated to ERK1 null mice to generate *ERK2*$^{fl/fl}$,*ERK1*$^{-/-}$,*αGSU:CRE*, hereafter designated DKO. Pituitary-specific ERK2 null males and females (with normal ERK1) were viable and fertile. They were born at expected Mendelian frequencies, and were grossly and histologically unremarkable at 3 months of age (data not shown). In contrast, at 3 weeks of age, DKO animals were represented at less than the expected frequency (expected: 0.5, observed: 0.24; n=327). Within litters of neonates, some DKO animals were frequently identified either dead, or as unthrifty pups that failed to nurse and that died shortly after birth.

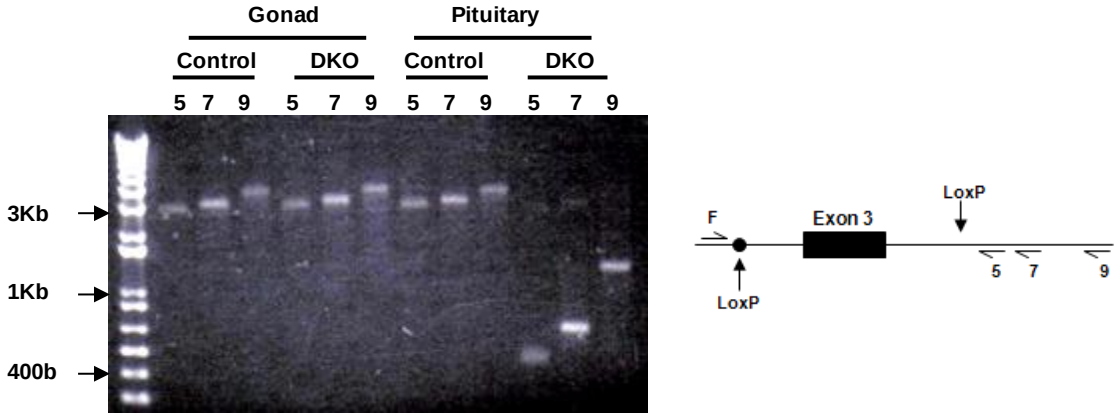
To validate the fidelity of αGSU:Cre -mediated recombination, we crossed *αGSU:Cre* males with Rosa26 reporter (R26R) females and assayed β-galactosidase activity in tissue lysates from both Cre-expressing, and non-expressing offspring. β-galactosidase activity was significantly increased in pituitary lysates from Cre-expressing animals as compared with Cre-negative controls (Figure 3.1A). Differences in β-galactosidase activity were not observed in lysates from other tissues (lung, kidney, heart and brain, Figure 3.1A and data not shown). These data support the pituitary-enriched activity of the αGSU:Cre. Cre-dependent recombination specifically at the *ERK2* locus was verified by PCR from *ERK2*$^{fl/fl}$,*CRE*$^{+}$ and *ERK2*$^{fl/fl}$,*CRE*$^{-}$ animals using primers flanking the floxed region of the *ERK2*

Figure 3.1. Validation of the ERK1/2 DKO mouse. (A) Pituitary-specific expression of the αGSU-Cre was assessed by crossing the αGSU-Cre transgenic male with R26R female mice and comparing Beta-galactosidase activity in tissue lysates from Cre-expressing and non-expressing (control) offspring. β-gal activity in individual samples was followed over a 60 minute time course. Symbols represent mean +/-SEM at each time point as measured in specified tissues from 3 animals. Asterices represent significant ($p<0.05$) differences between Cre-expressing and control samples at given time points. (B) Cre-mediated recombination at the *ERK2* locus within the pituitary was detected by PCR using genomic DNA from the specified tissues. For each sample, the forward primer designated 'F' was paired individually with reverse primers labeled '5', '7', and '9' spanning the floxed genomic region as indicated in the schematic. Lanes are labeled with the specified reverse primer used for the reaction. Molecular weight marker is shown in the left lane. Equivalent results were obtained from males and females. (C) Cell type-specific loss of ERK protein in the ERK1/2 DKO was determined by coimmunofluorescent labeling of pituitary sections from control (panels A-C) and DKO (panels D-F) male animals using FITC-conjugated antibodies against ERK1/2 and Texas Red-conjugated antibodies against LHβ. ERK (A,D), LHβ (B,E), and simultaneous visualization of both wavelengths (C,F) are shown. Arrows indicate representative LHβ positive cells with distinct lack of ERK labeling. Bars, 100 uM.

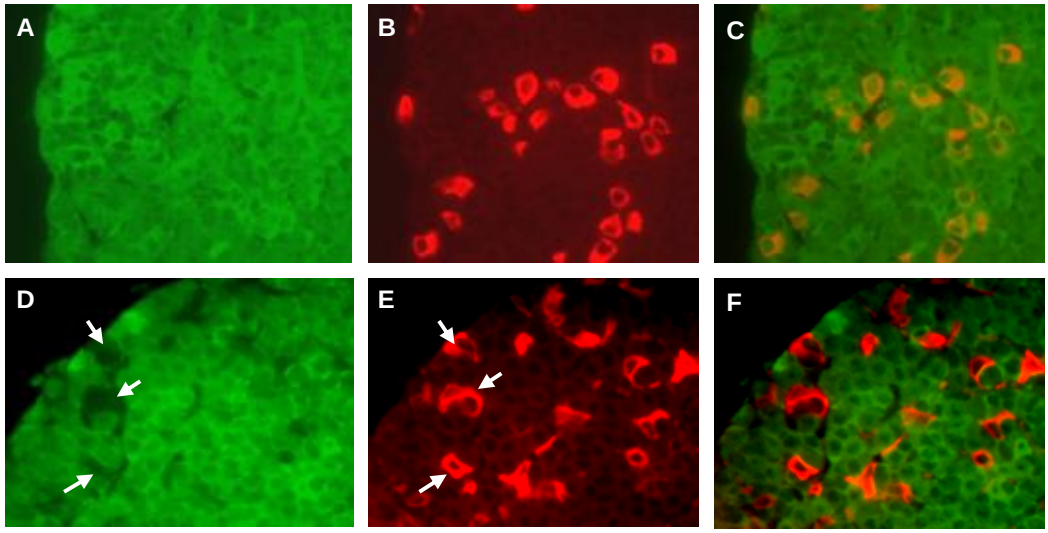
A.


control pituitary
CRE+ pituitary
Light units
Time (min)
control lung
CRE+ lung
control gonad
CRE+ gonad

B.


Gonad
Pituitary
Control
DKO
Control
DKO
5 7 9 5 7 9 5 7 9 5 7 9
3Kb
1Kb
400b
F
Exon 3
LoxP
LoxP
5 7 9

C.


A
B
C
D
E
F

locus (Figure 3.1B, schematic). Products indicating excision of the floxed region of the *ERK2* gene were only produced from pituitary genomic DNA of Cre^+ individuals, indicating tissue-specific susceptibility of the *ERK2* locus to Cre-mediated recombination (Figure 3.1B, left panel).

Immunohistochemical co-labeling of pituitary sections from adult DKO and control animals using antibodies against ERK2 and LHβ confirmed a high level of co-localization between LHβ and negative ERK labeling, indicating that ERK deficiency was relatively penetrant within the gonadotropes of DKO animals (Figure 3.1C). Immunohistochemical identification of TSHβ within ERK-deficient, LHβ negative cells in these sections confirmed the prediction that αGSU-expressing thyrotropes were also rendered ERK-deficient within this line (data not shown). Overall, these results support the fidelity of the compound genetic deletion within the adult DKO animals.

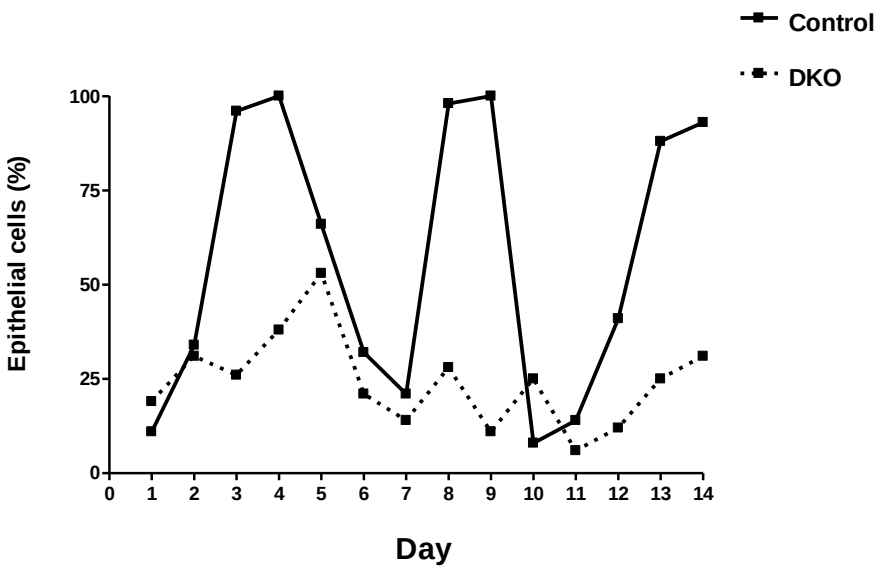
Phenotypic characterization of the ERK1/2 DKO mouse

DKO animals of both genders grew at the same rate as Cre^- littermate controls, and were grossly indistinguishable from controls from birth through 8 months of age (data not shown). Test matings indicated that DKO males were fertile, while females showed no evidence of estrus cycle activity and did not mate. To further assess estrous cycle activity, vaginal cytology was performed in control and DKO mice. Cyclic cytological changes indicative of estrous cycle activity were observed in control mice (Figure 3.2A). In contrast, vaginal cytology in the DKO females showed a preponderance of polymorphonuclear leukocytes with occasional basal epithelial cells over the course of the study, consistent with anestrous behavior.

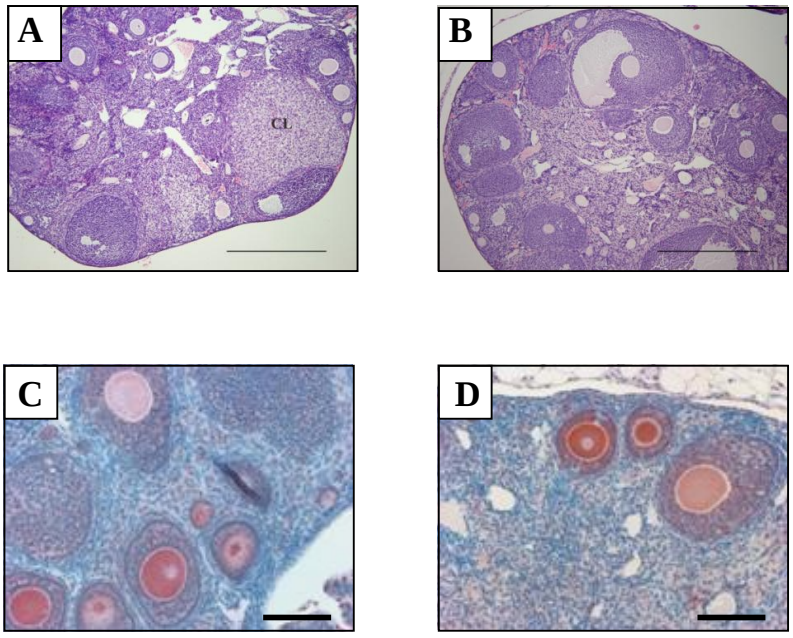
Histological examination of a variety of tissues including the reproductive tract from DKO and control males revealed no abnormalities. In contrast, ovaries from

Figure 3.2. Assessment of estrous cycle activity in the ERK1/2 DKO mouse. (A) Vaginal cytology was performed at 24 hour intervals by microscopic examination of Wright's stained vaginal lavage effluents. One-hundred cells were counted and the percentage of epithelial cells at each time point was determined. Data are shown for a single animal; however, similar results were obtained from 3 animals of each genotype. (B) Hematoxylin and eosin stained histological sections of ovaries from control (panel A) and DKO (panel B) animals are shown. A representative corpus luteum is indicated (CL) Bars in A and B, 200 μM. Microscopic sections from control (panel C), and DKO (panel D) ovaries were immunohistochemically stained using an antibody against ERK1/2. Both control and DKO animals were ERK1 null, thus all specific labeling represents ERK2 protein. Bars in C and D, 100 μM.

A.


Control
DKO
Epithelial cells (%)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day

B.


A
CL
B
C
D

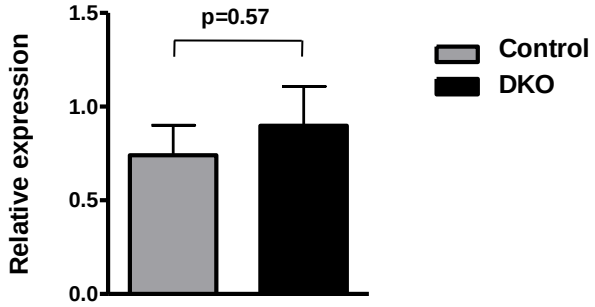
DKO females contained follicles at various stages of maturation (primary through large antral follicles), but were specifically devoid of luteal tissue, while ovaries from control females were histologically unremarkable (Figure 3.2B, panels A and B). The combined data from cytological analysis of estrous cycle behavior and histological evidence of lack of luteal tissue within the ovary in DKO female mice supported the preliminary conclusion that the DKO mice were anovulatory. To address whether the ovarian lesion in DKO animals may reflect promiscuous Cre expression and lack of ERK2 protein in the ovary, we examined ERK2 expression in ovaries from DKO and control females immunohistochemically. Expression of ERK2 was observed in both DKO and control ovaries indicating that the anovulatory ovarian lesion was secondary to the primary pituitary defect (Figure 3.2B, panels C and D).

ERK1/2 DKO mice are euthyroid

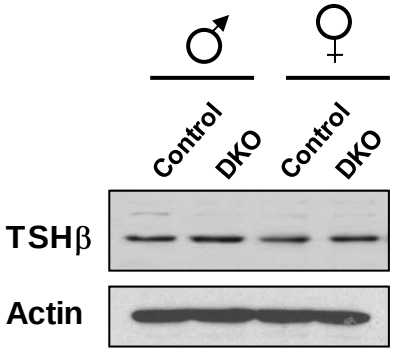
Since αGSU is expressed in thyrotropes as well as in gonadotropes, our model would predict fully penetrant Cre-dependent disruption of ERK signaling in the thyrotropes of DKO animals. To evaluate thyroid function in these animals, we used quantitative PCR (qPCR) to measure whole pituitary content of TSHβ mRNA in DKO and control animals. Pituitary content of TSHβ protein was examined by immunoblot analysis and T4 levels in serum were examined using an ELISA. Pituitary TSHβ mRNA, TSHβ protein in whole pituitary lysates or serum T4 concentrations did not differ between DKO and control mice (Figure 3.3). These results confirm that DKO animals that survived to adulthood were euthyroid and effectively rule out thyroid dysfunction as a contributing factor to the anovulatory phenotype.

Figure 3.3. Evaluation of thyroid function in the ERK1/2 DKO mouse. (A) Relative transcript levels of *TSHβ* from whole pituitaries of female control and DKO animals were determined by quantitative PCR. Results were calibrated to levels of *TSHβ* mRNA from pituitaries of randomly cycling female wild type mice. Bars represent mean +/- SEM for 5 animals of each genotype. Means were compared by 2-tailed t-test. (B) Levels of TSHβ in whole pituitary lysates from control and DKO animals were compared by immunoblot. Actin is shown as a lane-loading control. (C) Serum levels of total T4 from randomly cycling wild type, control and DKO females were determined by ELISA. Bars represent mean +/- SEM for 3 (wild type) or 11 (control and DKO) animals in the respective groups. Means for control and DKO groups were compared by 2-tailed t-test.

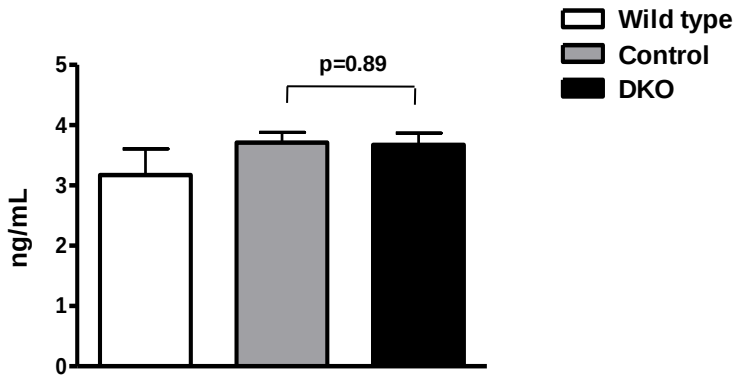
A.


p=0.57
Relative expression
1.5
1.0
0.5
0.0
Control
DKO

B.


♂
♀
Control
DKO
Control
DKO
TSHβ
Actin

C.


Wild type
Control
DKO
p=0.89
ng/mL
5
4
3
2
1
0

Expression of LHβ is reduced in pituitaries of ERK1/2 DKO animals

The lack of luteal tissue in the ovaries of DKO females suggests that DKO females were anovulatory and implicates a gonadotropin deficiency in these animals. To test this, we used quantitative PCR (qPCR) to measure transcript levels of the gonadotropin subunits (LHβ, FSHβ and αGSU), as well as the GnRH receptor (GnRHR) in the pituitaries of DKO and control animals. In females, LHβ mRNA levels were significantly lower in DKO's as compared with randomly cycling control animals (Figure 3.4A). In males, LHβ levels were not statistically significantly different between DKO and control animals (Figure 3.4A). Transcript levels of the other gonadotropin subunits, as well as the GnRHR did not differ between DKO and control animals for either gender (Figure 3.4A).

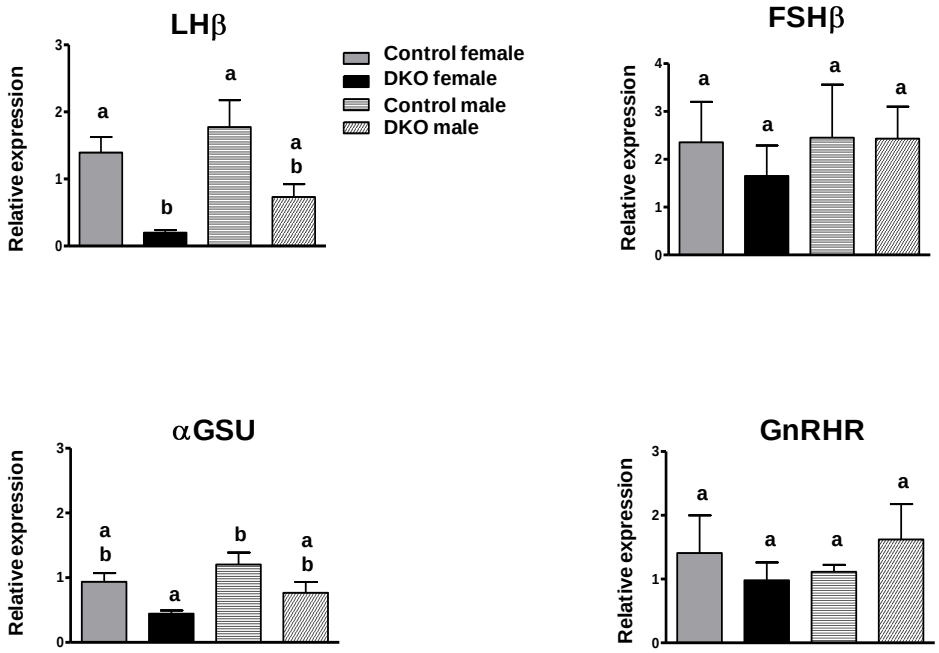
To assess the effects of ERK deletion on pituitary stores of gonadotropin subunits, we examined pituitary lysates by immunoblotting using antibodies against LHβ and FSHβ. LHβ was less abundant in pituitaries of DKO animals of both genders, as compared with controls, and was essentially undetectable in the pituitaries of DKO females (Figure 3.4B). Levels of FSHβ were similar between DKO and control animals of both genders (Figure 3.4B).

The anovulatory phenotype of ERK1/2 DKO females is rescued by pharmacological superovulation

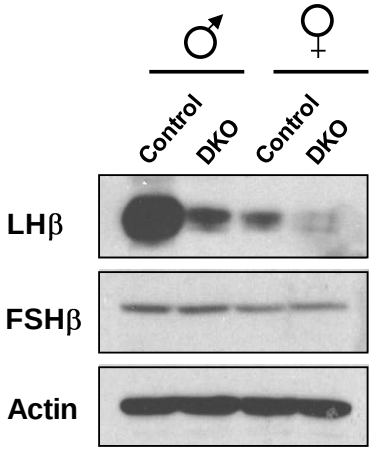
Our histological findings and gene profiling data suggest that the anovulatory infertility in the DKO mice was a direct consequence of LH deficiency. If correct, we reasoned that the ovaries of DKO females should remain capable of mounting an ovulatory response to an appropriate supra-ovarian gonadotropic stimulus. To test this, we subjected DKO and control females to conventional pharmacological

Figure 4. Basal expression of gonadotropin subunit and GnRHR genes in the ERK1/2 DKO mouse. (A) Whole pituitary relative transcript levels of the specified genes were determined by quantitative PCR. Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild type mice. Bars represent mean +/- SEM for 5 animals of each gender and genotype. Bars not sharing common letter designations represent mean values that are statistically significantly different ($p<0.001$). (B) Levels of LHβand FSHβ protein in whole pituitary lysates from control and DKO animals were compared by immunoblotting. Actin is shown as a lane-loading control.

A.


LHβ
Relative expression
Control female
DKO female
Control male
DKO male
FSHβ
αGSU
GnRHR

B.


♂
♀
Control
DKO
Control
DKO
LHβ
FSHβ
Actin

superovulation. Corpora lutea were present in the ovaries of gonadotropin-treated DKO animals albeit at a frequency (number of corpora lutea/ovary) less than that observed in superovulated control females (Figure 3.5). These results confirm the intrinsic ability of DKO ovaries to respond to an ovulatory signal.

ERK signaling is required for the response of the gonadotropin subunits following gonadectomy

Gonadal steroids regulate the expression of the gonadotropin subunit genes within the pituitary through negative feedback inhibition of GnRH release from the hypothalamus. Accordingly, gonadectomy leads to increased expression of the β subunits of LH and FSH through disinhibition of the hypothalamic GnRH system (13, 14). To determine the role of ERK signaling in mediating the gonadotrope response to endogenous GnRH hyperstimulation, we subjected control and DKO mice of both genders to gonadectomy. After 7 days, pituitary expression of the gonadotropin subunits and the GnRHR were assayed by qPCR. Following gonadectomy, significant increases in LHβ transcript levels were observed in control mice of both genders; however, the inductive effect of gonadectomy on LHβ transcript levels was blocked in DKO's (Figure 3.6A and B, upper left panels). In males, castration had no significant effect on FSHβ transcript levels in either DKOs or controls (Figure 3.6A, upper right panel). In contrast, ovariectomy led to an approximately 20-fold increase in FSHβ transcript levels in the control females (Figure 3.6B, upper right panel). In DKO females, ovariectomy led to a more modest elevation in FSHβ transcript levels, which did not reach statistical significance (Figure 3.6B, upper right panel). Gonadectomy led to significant increases in αGSU transcript levels in both control males and females; however, this response was blocked in DKO animals of both genders (Figure

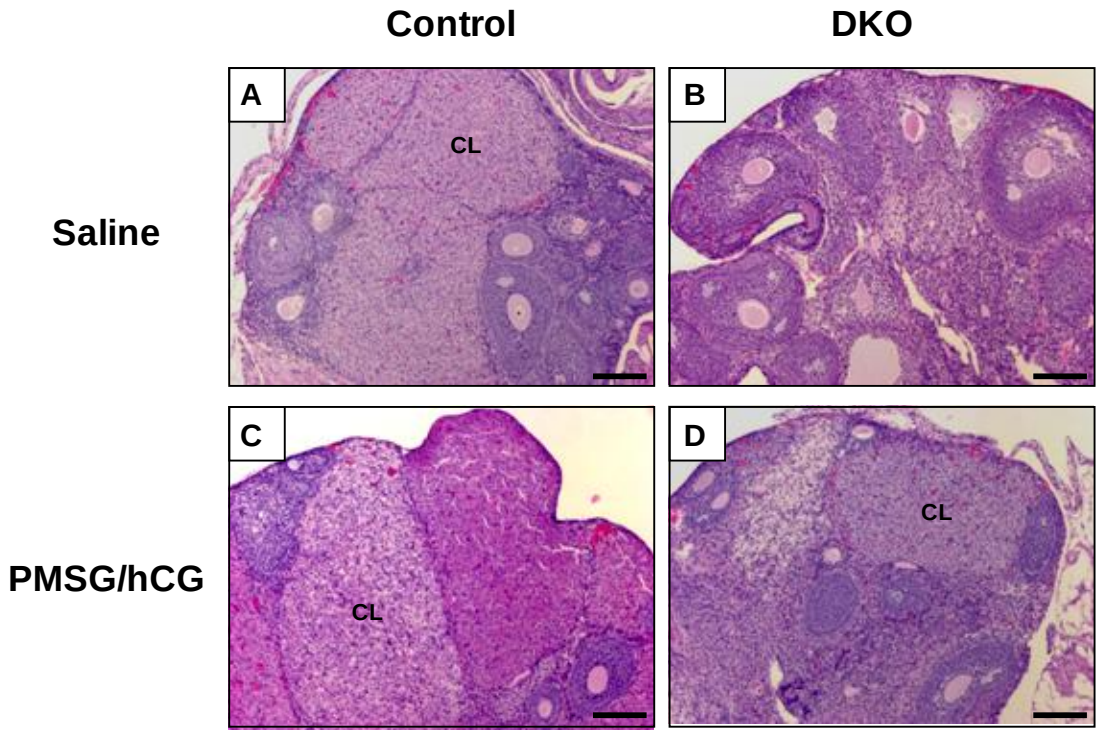
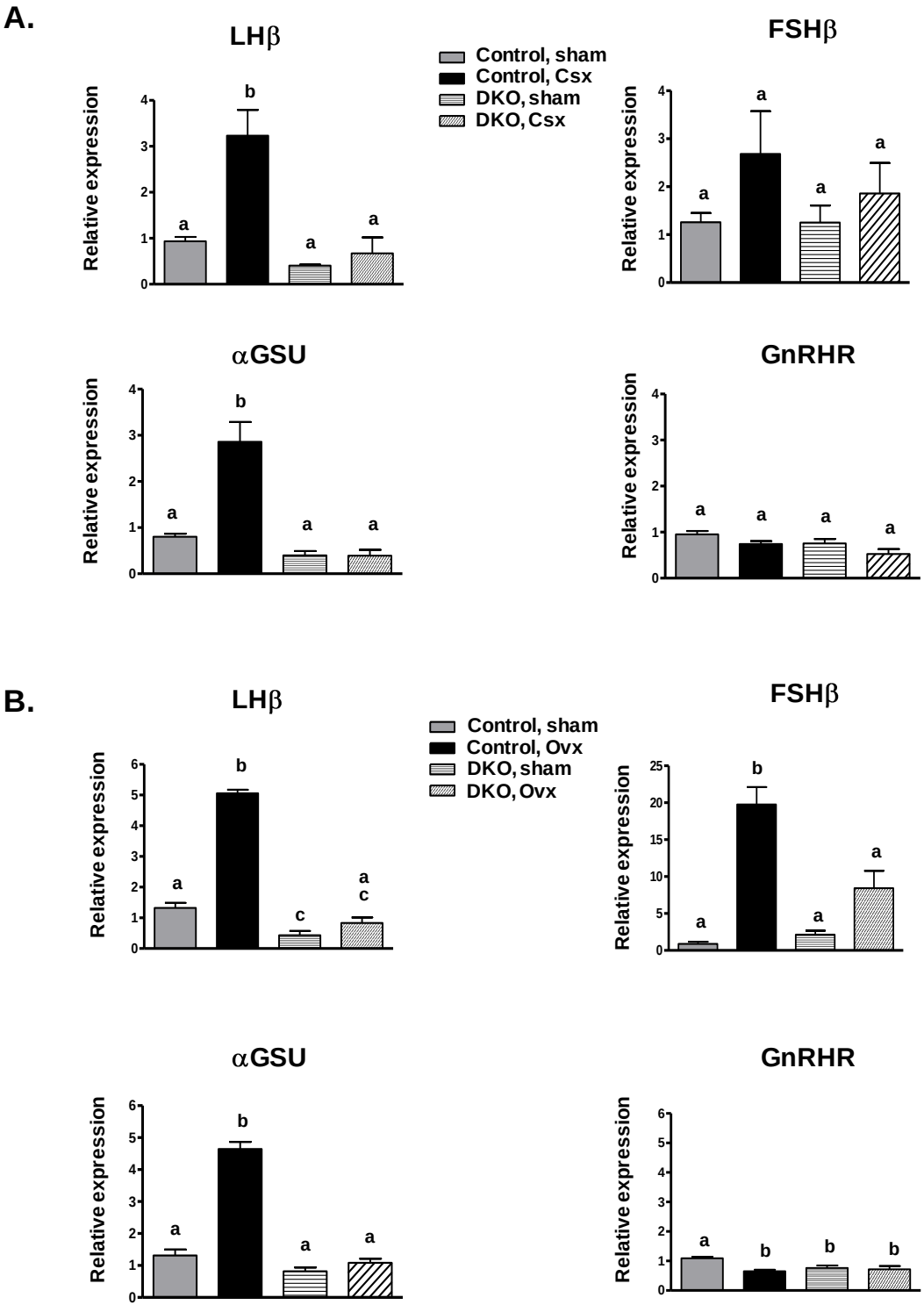


Figure 3.5. Pharmacological superovulation rescues the anovulatory phenotype of the ERK1/2 DKO mouse. Control and DKO females were injected intraperitoneally with vehicle or 5 units PSMG, followed in 48 hours by vehicle or 5 units of hCG. After 9 days, ovaries were collected and examined histologically. Ovarian sections from vehicle-treated (A,B) and superovulated (C,D) control (A,C) and DKO (B,D) animals are shown. Arrows indicate corpora lutea. Bars, 200 □M.

Figure 3.6. ERK1/2 DKO animals of both genders fail to upregulate LHβ following gonadectomy. Control and DKO animals were either castrated (Csx), ovariectomized (Ovx), or sham operated (sham). After 7 days, pituitaries were collected and analyzed by qPCR for relative transcript levels of the indicated gonadotropin subunit genes and the *GnRHR*. Data are shown separately for males (A) and females (B). Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild type mice. Bars represent mean +/- SEM for 6 males, or 7 females, per group, and represent pooled results from two separate experiments. Bars not sharing common letter designations represent mean values that are statistically significantly different ($p<0.001$). Note the differences in scaling of the Y-axis for the FSHβ results in the female.


A.
LHβ
FSHβ
αGSU
GnRHR
Control, sham
Control, Csx
DKO, sham
DKO, Csx
Relative expression
B.
LHβ
FSHβ
αGSU
GnRHR
Control, sham
Control, Ovx
DKO, sham
DKO, Ovx
Relative expression

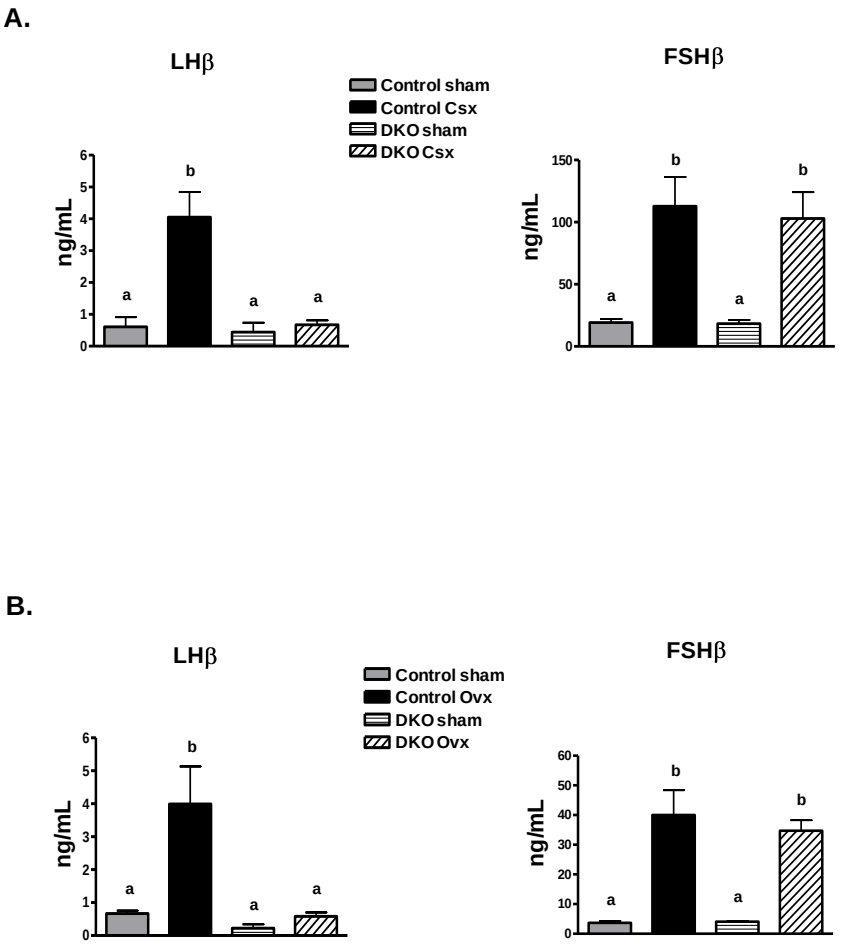


Figure 3.7. Serum gonadotropin levels do not increase in DKO animals of either gender following gonadectomy. Control and DKO animals were either castrated (Csx), ovariectomized (Ovx), or sham operated (sham). After 7 days, animals were euthanized and serum levels of LHβ and FSHβ were determined by ELISA. Data are shown separately for males (A) and females (B). Bars represent mean +/- SEM for 6 males, or 7 females, per group, and represent pooled results from two separate experiments. Bars not sharing common letter designations represent mean values that are statistically significantly different ($p<0.001$).

3.6A and B, lower left panels). Transcript levels of the GnRHR in males did not differ between either genotype or treatment, while in females, statistically significant but biologically insignificant differences were observed in GnRHR transcript levels between sham-operated control animals and all other groups (Figure 3.6 A and B, lower right panels).

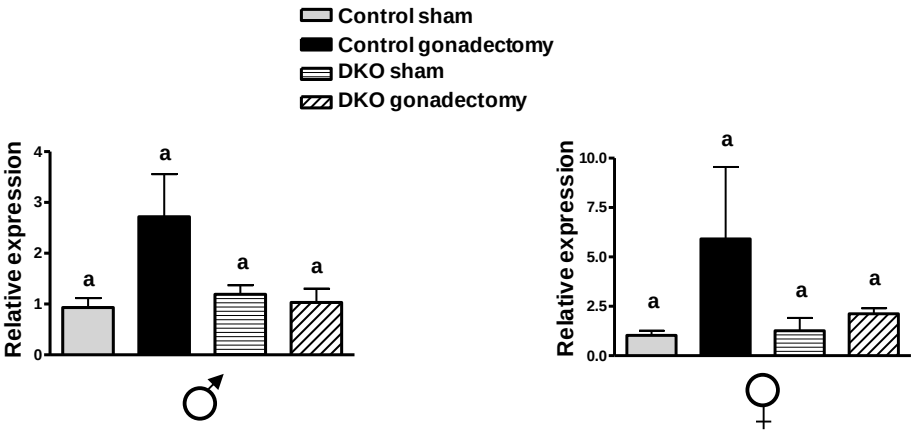
In parallel to the gene profiling results, serum levels of both LHβ and FSHβ rose significantly following gonadectomy in control animals of both genders (Figure 3.7A and B, left panels). Baseline serum FSHβ levels were not different between sham operated DKO and control animals of either gender. In contrast, baseline serum levels of LHβ were significantly lower in sham operated DKO's as compared to controls, and significant increases in serum LHβ did not occur following gonadectomy in DKO animals of either gender. These results support the conclusion that ERK signaling plays a critical role in mediating upregulation of both subunits of LH in response to GnRH stimulation in vivo.

ERK-deficient gonadotropes fail to upregulate the immediate early response gene *Egr1* following GnRH stimulation

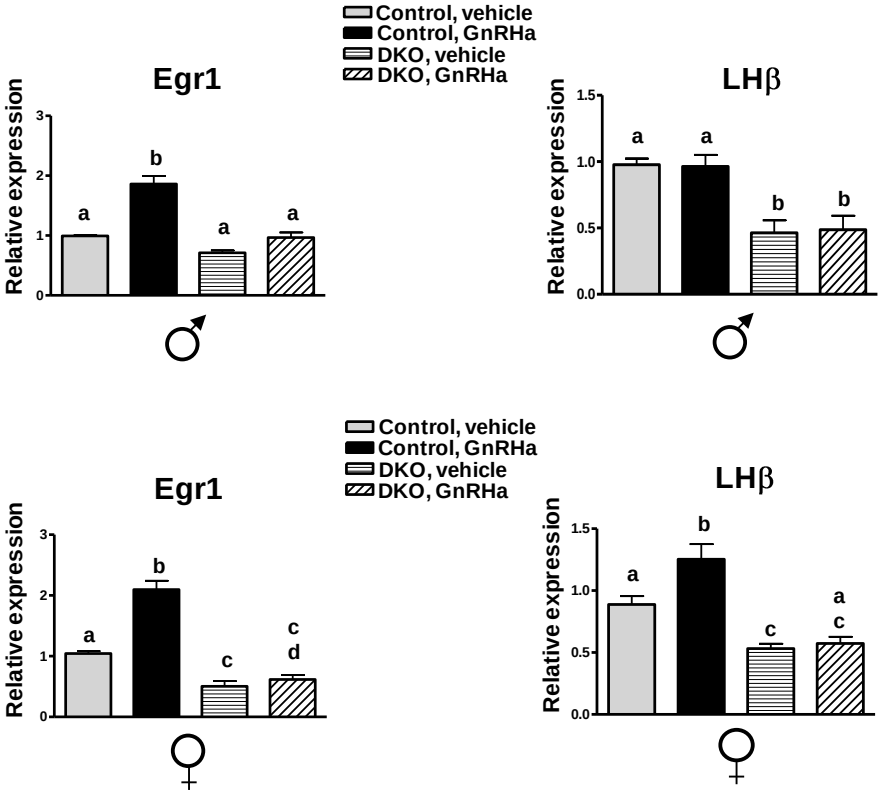
Early growth response factor-1 (EGR1) is a transcription factor encoded by an ERK-dependent immediate early gene that has been shown to play a key role in the transcriptional regulation of LHβ (15). To determine the effect of ERK ablation on the ability of gonadotropes to upregulate EGR1 in response to GnRH stimulation, we subjected control and DKO mice of both genders to gonadectomy and after 7 days, measured pituitary expression of *Egr1* by qPCR. In the control animals, mean *Egr1* transcript levels rose approximately 3-fold and 5-fold following gonadectomy in males and females, respectively; however, due to the high variability in transcript levels observed in the gonadectomized animals, these differences were not statistically

Figure 3.8. ERK-deficient gonadotropes fail to upregulate the immediate early gene Egr1 in response to GnRH stimulation. (A) Control and DKO animals of both genders were either gonadectomized (gonadectomy), or sham operated (sham). After 7 days, pituitaries were collected and analyzed by qPCR for relative transcript levels of *Egr1*. Data are shown separately for males (left panel) and females (right panel). Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild type mice. Bars represent mean +/- SEM for 6 animals per group, and represent pooled results from two separate experiments for each gender. Bars not sharing common letter designations represent mean values that are statistically significantly different. (B) Pituitaries from control and DKO animals were dispersed into primary culture (2 pituitaries/well) and treated for 20 minutes with vehicle or with 100 nM of the GnRH agonist buserelin (GnRHa). Relative transcript levels of *Egr1* and *LHβ* were measured in each sample by qPCR. Bars represent mean +/- SEM for 6 wells representing pooled results from 2 separate experiments. Data are shown separately for males (upper panels) and females (lower panels). Bars not sharing common letter designations represent mean values that are statistically significantly different ($p \leq 0.05$).

A.


Control sham
Control gonadectomy
DKO sham
DKO gonadectomy
Relative expression
a
a
a
a
♂
Relative expression
a
a
a
a
♀

B.


Control, vehicle
Control, GnRHa
DKO, vehicle
DKO, GnRHa
Egr1
Relative expression
a
b
a
a
♂
LHβ
Relative expression
a
a
b
b
♂
Egr1
Relative expression
a
b
c
c
d
♀
LHβ
Relative expression
a
b
c
a
c
♀

significantly different (Figure 3.8A). No differences were observed in Egr1 transcript levels following gonadectomy in DKO animals of either gender (Figure 3.8A).

Given the dynamic nature of the *Egr1* transcriptional response to GnRH stimulation, we considered that the lack of statistically significant differences between the sham operated and gonadectomized control animals may reflect type II error. However, power analysis indicated that to achieve a power of 0.8 for detection of a fourfold difference in *Egr1* transcript levels within the context of this experimental design would require sample sizes of 8 and 71 animals per group, for males and females, respectively. To circumvent this limitation, we undertook an alternative strategy and measured *Egr1* and *LHβ* transcript levels by qPCR in primary pituitary cell cultures from DKO and control animals following stimulation with vehicle or the GnRH agonist buserelin (GnRHa). We reasoned that this experimental approach would allow synchronization of experimental units with respect to the onset and duration of the GnRH signal in a manner that cannot be accomplished within our in vivo paradigm. Baseline *Egr1* transcript levels were significantly reduced in cells from DKO females as compared to controls (Figure 3.8B, lower left panel). *Egr1* transcripts were significantly upregulated following GnRHa exposure in cells from control animals of both genders; however, upregulation of *Egr1* mRNA was not observed in cells from DKO animals of either gender (Figure 3.8B, left panels). *LHβ* transcripts were significantly reduced in cultures of vehicle-treated cells from DKO animals of both genders, as compared with controls. Following GnRH stimulation, a statistically significant, but quantitatively negligible increase in *LHβ* transcripts was observed in cells from control females. Overall, the reduction in GnRH-induced *Egr1* transcription in ERK-deficient gonadotropes underscores a key mechanism linking ERK signaling to LH biosynthesis and highlights the unique importance of the ERK pathway for female fertility.

DISCUSSION

Using a genetic approach, we show here that ablation of ERK1 and 2 in the anterior pituitary leads to infertility in females, but not males. The anovulatory infertility in the ERK-deficient females in this study was associated with marked reductions in pituitary levels of LHβ. Our studies on the pituitary response to gonadectomy indicate further that ERK-deficient gonadotropes are incapable of transcriptional upregulation of either LHβ or αGSU following pulsatile hyperstimulation by hypothalamic GnRH within the intact endocrine milieu of the living animal. Thyroid function remained normal in animals with pituitary ERK deficiency suggesting that the function of the thyroid axis does not depend upon ERK signaling at the level of the thyrotrope.

While our model reveals that ERK signaling is absolutely required for appropriate LH biosynthesis, our data also suggest that the ERK pathway may play a partial role in mediating transcriptional upregulation of FSHβ in the female. Nevertheless, our observations of whole pituitary and serum FSH levels would suggest that overall expression of FSHβ is largely ERK-independent. Moreover, the presence of antral follicles in the ovaries of the DKO females, along with the absence of corpora lutea, strongly implicates LH deficiency as the primary cause of infertility in these animals, and further supports the relative ERK-independence of FSH biosynthesis. This is further corroborated by the observation that the anovulatory phenotype was able to be rescued in the DKO females by administration of exogenous gonadotropin receptor ligands. Our model thus confirms the ERK pathway as a dominant link between the activation of the GnRHR and LHβ biosynthesis within the gonadotrope in vivo. Gender differences in the transcriptional response of the gonadotropin subunits to GnRH stimulation have been reported previously (16-19); however, the sexually dimorphic nature of the requirement for the ERK signaling

pathway that is disclosed by our model is a novel observation that has not previously been appreciated.

In contrast to the reproductive phenotype displayed within the adult DKO females, the majority of DKO's predicted to occur within this pedigree remained unaccounted for at 3 weeks of age. The regular finding of dead DKO neonates suggests that pre- or perinatal mortality may represent a distinct phenotype resulting from pituitary-targeted disruption of the ERK pathway. Perinatal mortality has been described following deletion of several genes that interfere with appropriate development of anterior pituitary cell lineages (20-22). The pathophysiological basis of perinatal mortality has perhaps been most clearly defined in the Prop1 deficient mouse, in which severe congenital hypothyroidism secondary to hypoplasia of the Pit1-dependent thyrotrope lineage leads to surfactant deficiency and respiratory failure in neonatal pups (23). Whether ERK signaling plays a role in thyrotrope development through modification of either the expression or function of Prop1 or Pit1 is unknown. However, as previously reported, some variability has been observed in the timing of onset and penetrance of αGSU-Cre-mediated target gene recombination within the developing pituitary (24). Therefore, it is intriguing to hypothesize that the perinatal mortality observed in our model may reflect early onset Cre expression within a subset of DKO animals, leading to more extensive loss of ERK activity throughout the pituitary primordium, and disruption of critical ERK-dependent processes of pituitary cell lineage specification. Studies are currently underway to define the underlying mechanism of perinatal mortality within this model, and to clarify the role of the ERK pathway in development of the Pit1-dependent anterior pituitary cell lineages.

The transcriptional regulation of *LHβ* has been studied in a variety of experimental systems (15, 25, 26). The *LHβ* promoter contains binding sites for several transcription factors, including the zinc-finger transcription factor EGR1 (15,

27). EGR1 has been shown to play a critical role in *LHβ* transcription and appears to function primarily as an amplifier of *LHβ* promoter activity, contributing to the responsiveness of the promoter to changes in the amplitude or pulse frequency of GnRH stimulation (26, 28). Interestingly, despite the broad pattern of expression of EGR1, the predominant phenotype of the EGR1 null mouse consists of female anovulatory infertility associated with LH deficiency (29). The phenotype of the female ERK DKO mouse reported here bears a striking similarity to that of the EGR1 null, and the inability of ERK-deficient gonadotropes to upregulate EGR1 in response to GnRH stimulation indicates that ERK-dependent induction of EGR1 is a dominant mechanism by which GnRHR occupancy is linked to transcription of the *LHβ* gene in vivo. However, whether the LH deficiency in the ERK DKO is due solely to a failure of Egr1 induction, or whether the phenotype may also reflect a lack of ERK-dependent EGR1 phosphorylation is not clear. For example, in one study, the GnRH-induced transcriptional activity of constitutively expressed Egr1 was attenuated in transiently transfected gonadotrope cells by treatment with the MEK inhibitor PD98059 (30). This observation raises the possibility that the ERK pathway may modulate LHβ gene transcription at multiple levels involving not only gene induction but also direct phosphorylation of LHβ transactivators such as EGR1. Ultimately, full clarification of the role of ERK activation in LH biosynthesis will require more comprehensive genomic and proteomic approaches aimed at identification of the entire complement of ERK-dependent genes that are expressed in the gonadotrope, as well as relevant targets of ERK-dependent phosphorylation.

In contrast to the females, ERK DKO males retained normal reproductive function and gonadal histology. This observation provides further interesting comparison with the EGR1 null mouse as EGR1 null males are also reproductively competent (29). However, despite their fertility, EGR1 null males did display Leydig

cell atrophy while testicular histology in the ERK-deficient males in this study was unremarkable (29). The testicular lesion in the EGR1 null male undoubtedly reflects a more severe impairment of LHβ production than in the ERK DKO. Thus, despite the measurable decrease in pituitary LHβ protein content in the DKO males in this study, production of LH in these animals was clearly sufficient to maintain normal testicular function and structure as well as fertility. The inability of gonadotropes from ERK-deficient males to upregulate EGR1 in response to GnRH stimulation suggests further that basal EGR1 expression is sufficient to support this level of LHβ production. Overall, our data support a model in which the ERK pathway functions in both genders as a dominant link between the GnRHR and the *Egr1* locus. In turn, EGR1 serves as a primary mediator of GnRH-induced upregulation of LHβ. The gender difference in the requirement for ERK signaling in the gonadotrope would thus seem to reflect the relatively modest levels of LH that are required to support Leydig cell function in the male, as compared to the high levels of LH that are required for ovulation in the female. Consistent with that notion, transcriptional regulation of the gonadotropin subunit genes is highly dependent upon the frequency of the pulsatile hypothalamic GnRH signal; rapid pulsatility favors LHβ production while slower GnRH pulsing appears to favor production of FSHβ (31). Moreover, studies using the LβT2 cell model indicate that the ability of the gonadotrope to interpret variations in the GnRH interpulse interval may depend upon a functional ERK signaling module (32). During the estrous cycle, regular variations occur in the frequency of GnRH pulsatility; pulsatility increases markedly during proestrus eventually leading to a preovulatory GnRH/LH surge and ovulation. We propose that the ERK module plays a central role in the ability of the gonadotrope to respond to variations in the pulse pattern of the GnRH signal thereby linking changes in GnRH pulse frequency with the coordinated physiological outcomes associated with the female reproductive cycle.

The fact that males have no requirement for cyclic variations in GnRH pulsatility is consistent with our observations that steroidogenesis and spermatogenesis are unaffected by disruption of the ERK signaling pathway at the level of the gonadotrope.

In summary, we have shown that ablation of the ERK signaling pathway in pituitary gonadotropes leads to infertility in female but not male mice. Our data establish an essential role for the ERK pathway in mediating transcriptional upregulation of LH in the gonadotrope following GnRH stimulation in vivo, and lend insight into the molecular mechanisms underlying sexually dimorphic control of reproductive function.

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CHAPTER 4

ERK SIGNALING, BUT NOT C-RAF, IS REQUIRED FOR GONADOTROPIN-RELEASING HORMONE (GNRH) INDUCED UPREGULATION OF NUR77 IN PITUITARY GONADOTROPES

SUMMARY

Stimulation of pituitary gonadotropes by hypothalamic GnRH leads to the rapid expression of several immediate early genes that play key roles in orchestrating the response of the gonadotrope to the hypothalamic signal. Elucidation of the signaling mechanisms that couple the GnRH receptor to this immediate early gene repertoire is critical for understanding the molecular basis of GnRH action. Here we identify the orphan nuclear receptor Nur77 as a GnRH-responsive immediate early gene in αT3-1 cells as well as in mouse gonadotropes in vivo. Using a variety of molecular biological methods, we show that GnRH-induced transcriptional upregulation of *Nur77* in αT3-1 cells is dependent upon ERK signaling, but that neither activation of the ERK pathway, nor the transcriptional response of *Nur77* to GnRH requires the activity of c-Raf. In corroboration of these results, gonadotropes from mice with pituitary deficiency of ERK signaling failed to upregulate *Nur77* following GnRH stimulation. In contrast, *Nur77* responsiveness to GnRH was maintained in gonadotropes from mice with pituitary targeted ablation of c-Raf. These results further clarify the role of ERK signaling in regulation of the GnRH-induced immediate early gene program in the gonadotrope, and shed new light on the complex functional organization of this signaling pathway in these cells.

INTRODUCTION

In mammals, reproductive function is dependent upon the coordinated synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary gonadotrope. Production of the gonadotropins is largely controlled by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH). GnRH is released in pulsatile fashion from the hypothalamus and acts through the gonadotropin-releasing hormone receptor (GnRHR), to stimulate biosynthesis of the gonadotropin subunits, as well as the GnRHR itself. The signaling events initiated by the GnRHR coordinate the expression of a diverse set of immediate early response genes, several of which have been shown to regulate gonadotropin biosynthesis (1-4). In the gonadotrope, as in most other cell types, early response genes play a critical role in linking a relatively transitory extracellular stimulus (the pulsatile GnRH signal) with more sustained changes in gene expression that underlie physiologically appropriate cellular responses to that stimulus (gonadotropin biosynthesis). Elucidation of the signaling activities that link the GnRH signal with the immediate early gene repertoire is thus important for understanding the molecular basis of gonadotrope function.

The ERK signaling pathway is rapidly activated by GnRH and ERK activity has been linked to the expression of several genes important for gonadotrope function including the gonadotropin subunit genes, as well as the dual specificity MAP kinase phosphatase *MKP2/DUSP4* (1, 5-8). ERK activity may also play a role in homologous regulation of the *GnRHR* (9). Several ERK-dependent immediate early genes have been shown to play key roles in mediating the effects of GnRH, including Early growth response protein 1 (*Egr1*), *c-Fos*, and Activating transcription factor 3 (*ATF3*) (1, 4-6).

Nur77 (NR4A1, NGFIB, NAK1, TR3) is an immediate early gene belonging to the NR4A family of orphan nuclear receptors. Nur77 is rapidly upregulated in response to a wide range of extracellular signals and has been shown to play diverse and important roles as a transcriptional regulator in several cell types with high metabolic demands including neurons, skeletal and cardiac myocytes, hepatocytes and adipocytes (10-13). Nur77 has also been shown to regulate the response of macrophages and endothelium to various pro-inflammatory cytokines and plays additional roles within the hypothalamic-pituitary-adrenal axis in the regulation of pro-opiomelanocortin gene transcription in pituitary corticotropes, as well as in adrenal and gonadal steroidogenesis (14-18). Microarray analysis showed that Nur77 was strongly upregulated by GnRH in the murine gonadotrope-derived LβT2 cell line (19, 20). Nur77 was also shown to be expressed in the less differentiated αT3-1 gonadotrope cell line; however, to our knowledge, the signaling mechanisms underlying GnRH induction of Nur77 have not been examined in detail either in this model or in vivo (21). ERK activity has been shown to be important for agonist-induced upregulation of Nur77 in several cell types (22-26). Therefore, we set out to test the specific hypothesis that ERK signaling is required for GnRH-induced expression of Nur77 in the gonadotrope. Our results establish Nur77 as an ERK-dependent GnRH-responsive immediate early gene in vivo and shed unexpected new light on the functional organization of the ERK pathway in the gonadotrope.

MATERIALS AND METHODS

Cells, reagents, and animals:

αT3-1 cells were a generous gift from Dr. Pamela Mellon (University of California at San Diego, San Diego, CA) and were cultured as described previously (27). NIH 3T3 cells were a gift from Dr. Joseph Wakshlag (Cornell University,

Ithaca, NY) and were cultured as described previously (28). Primary antibodies against Nur77 (rabbit polyclonal), ERK2, c-Raf, B-raf and β-actin, along with horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated anti-rabbit secondary antibody for immunocytochemistry was obtained from Vector Laboratories (Burlingame, CA). Anti-phospho-ERK was from Sigma Chemical (St Louis, MO). Mouse monoclonal anti-Nur77 antibody was from Pharmingen (San Diego, CA). Rabbit anti-LHβ antibody was from the National Hormone and Peptide Program; NIDDK. Rat monoclonal anti-HA antibody was from Roche (Indianapolis, IN). Buserelin (des-GLY^{10} [D-Ser(t-But)^{6}]-LH-RH Ethylamide; referred to as GnRHa), corticotrophin-releasing hormone (CRH), and the GnRHR antagonist antide, were from Sigma (St. Louis, MO). All other pharmacological inhibitors were from Calbiochem (Gibbstown, NJ).

The c-Raf floxed mouse and the ERK1/2 pituitary targeted compound knockout mouse have been described previously (29, 30). αGSU-CRE mice, in which Cre recombinase is expressed under regulatory control of a 4.6 kb fragment of the mouse αGSU promoter, were purchased from Jackson Laboratories (Bar Harbor, ME). Swiss Webster mice used as wild type were purchased from Taconic Farms (Germantown, NY). Animals were maintained and treated in compliance with the specifications of the Cornell University Institutional Animal Care and Use Committee. Genotyping of the c-Raf conditional knockout animals was performed by PCR using primers 5'- AGCCGAGTCAGCAAATGCACTG – 3', and 5' – AGTAGTCTACGGCACGTTTACTAGG – 3'. Genomic analysis of Cre-dependent recombination at the *c-Raf* locus was performed by PCR using primers "Pr. F" 5'-AGCCGAGTCAGCAAATGCACTG-3', "Pr. 3" 5'-

TATCACCTGCCAGGAACCAACAAGC-3', and "Pr. 4" 5'-AAGCCAACACTGCTCACTGTGTGGC-3'.

Plasmid construction and transfection

The pKH3 expression vector, containing three tandem copies of the hemaglutinin (HA) epitope tag, was a gift from Dr. Jun-lin Guan. The Raf-CAAX expression vector was a gift from Dr. Linda Van Aelst (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). An expression vector for the c-Raf-S375M catalytic mutant fused to GFP was a gift from Dr. Guillermo Romero (University of Pittsburgh, Pittsburgh, PA). This mutant Raf cDNA was subcloned without the GFP coding sequence into pKH3 as a BamHI/EcoRI fragment using standard methods to generate pKH3-RafS375A. To generate the Nur77 luciferase reporter, a 1566 bp fragment of the Nur77 proximal promoter was amplified by PCR from mouse genomic DNA using primers 5'- GGTTAGTCTGCTGCACTGGTG – 3' and 5'-AAGCTTCAGTCGCAGCCTGGTCC – 3' and subcloned as an SSTI-HindIII fragment into the luciferase expression vector Luclink2 using standard methods. The c-Fos luciferase reporter has been described previously (31). The fidelity of all constructs was verified by direct nucleotide sequencing. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Immunocytochemistry and fluorescence microscopy

For immunofluorescent co-labeling of ERK2 and LHβ, 5 μM pituitary sections were deparaffinized in xylene, rehydrated through EtOH dilution series to distilled H_20. Antigen retrieval was performed by boiling slides in 0.01M Citrate buffer (pH 6.0). Sections were washed in PBS, and blocked with 10% normal rabbit serum/10%

nonfat dry milk in PBS for 20 minutes at room temperature. Sections were then incubated in polyclonal anti-Nur77 primary antibody diluted 1:10 in PBS/1X casein (Vector), for two hours at 37^{o}C. Sections were further washed in PBS, and incubated at room temperature for 20 minutes with biotinylated rabbit-anti-goat IgG (Invitrogen). Normal rabbit IgG was used at equivalent concentration (ug/mL) as negative control. After incubation with biotinylated donkey-anti-rabbit IgG and further washing, sections were incubated with Streptavidin Alexa Fluor® 488 (Invitrogen) for 20 minutes at room temperature in the dark. Stained slides were washed further with PBS and stored in distilled water overnight at 4^{o}C. On day two, sections were blocked with Fab Fragment goat anti-rabbit IgG H&L (Jackson ImmunoResearch, West Grove, PA) diluted 1:50 in PBS for 30 minutes at 37^{o}C, and reblocked for 20 minutes with 10% goat serum/2Xcasein (Vector) in PBS. Rabbit anti-LHβ primary antibody (National Hormone and Peptide Program; NIDDK) was reconstituted in PBS at a concentration of 1 μg/μL, and applied at a 1:50 dilution for 2 hours at 37^{o}C, substituting normal rabbit IgG (Vector) at an equivalent concentration (ug/mL) as a negative control. LHb was detected with Texas Red goat-anti-rabbit IgG H&L (Vector) at a 1:80 dilution in PBS for 20 minutes at room temperature in the dark. Slides were washed and mounted in Vectashield DAPI (Vector Laboratories, Burlingame, CA). Images were obtained on a Nikon E400 epi-fluorescence microscope using the appropriate filters.

Luciferase assay

αT3-1 cells were seeded onto 1.5 cm dishes at approximately 60% confluence and were transfected and treated as indicated within 24 hours of plating. Media was removed and the cells were collected in 200 uL of 1X passive lysis buffer (Promega,Madison, WI). Cells were lysed by 2 freeze-thaw cycles and the lysates

were clarified by centrifugation. Protein concentration of the lysates was determined by Bradford assay, and lysates were adjusted to a concentration of 2 ug/uL. Aliquots (50 uL, 100 ug) were assayed for luciferase activity as previously described. Assays were run in triplicate for each treatment, and all experiments were repeated at least three times.

siRNA

Knockdown of c-Raf was performed in αT3-1 cells by direct transfection of siRNAs. To generate the siRNA's, an approximately 250 bp fragment from the 5' region of the c-Raf cDNA was generated by PCR using primers 5' – GCTTGGAAGACGATCAGC – 3' and 5' – TGCTGGGAACTAGCAGGC – 3'. This PCR fragment was sequenced to ensure fidelity, and then used as a template for the generation of a pool of mixed siRNA's using a commercial kit according to the manufacturer's instructions (Dicer kit, Invitrogen, Carlsbad, CA) Control siRNA's against LacZ were prepared in similar manner according to the kit instructions. Cells were plated in 1.5 cm dishes at approximately 40% confluence and were transfected the next day for 4 hours with 100 ng of siRNA per dish using Lipofectamine 2000 according to the manufacturer's instructions. Transfections were then repeated the following day. Treatments and preparation of lysates were performed 48 hours after the second transfection.

Immunoprecipitation and immunoblotting

For immunoblotting, cells were washed in cold PBS and scraped into PBS containing 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine. Cells were pelleted by centrifugation, and pellets were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 10% glycerol, 1%

Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM benzamidine. Lysates were cleared by centrifugation and protein concentrations of the lysates were determined by Bradford assay. Protein samples were boiled in SDS load buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then incubated with primary antibodies overnight in TBST with 5% milk. Membranes were then washed in TBST and incubated in HRP-conjugated secondary antibodies for one hour at room temperature. After further washing, proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer, Boston, MA).

For immunoprecipitations, cells were washed and lysed as above, except that the lysis buffer was adjusted to 200 mM NaCl. Aliquots of lysate containing 400 ug protein were adjusted to 5 mM Tris-HCl (pH 8.0), 70 mM NaCl, 5% glycerol, 0.2% Nonidet P-40, 0.01% SDS, 0.1% deoxycholate, 1 mM EDTA, 5 mM sodium vanadate, 0.2 mM PMSF, and 5 mM benzamidine in a total volume of 400 uL. Rabbit polyclonal anti-Nur77 antibody (2 ug/400 ug cellular protein) was added and the samples were rocked at 4^{o}C overnight. Protein A/G agarose (30 uL of 50% slurry per sample) was added and the samples were rocked at 4^{o}C for an additional hour. Beads were washed 3 times in wash buffer (5 mM Tris-HCl [pH 8.0], 70 mM NaCl, 1% glycerol, 0.1% Nonidet P-40, 0.01% SDS, 0.1% deoxycholate, 1 mM EDTA), and boiled in SDS load buffer. Immunoprecipitates were blotted as described above. After blocking, membranes were incubated with mouse monoclonal anti-Nur77 antibody at 1:1000 dilution in TBST with 5% milk overnight at 4^{o}C. Membranes were then washed and developed as described above.

Primary pituitary cell culture

Immediately following euthanasia, pituitaries were collected into cold DMEM containing 10% FBS. Pituitaries were digested at 37^{o}C for 10 minutes in DMEM containing 0.5 mg/mL each of collagenase and hyaluronidase (Sigma Chemical, St Louis, MO). Tissues were dispersed by repeated pipetting and tissue remnants were allowed to settle by gravity. The supernatant was removed and adjusted to 20% FBS. Remaining tissue was resuspended in DMEM containing 0.25 mg/mL each of the same enzymes, and digested for an additional 10 minutes. Following additional dispersal, the supernatants were combined. Cells were washed in DMEM containing 10% FBS, and plated in the same medium with Penicillin and Streptomycin on 10 mm diameter poly-L-lysine coated dishes at a density of 1 pituitary equivalent per well. Cells were maintained at 37^{o}C in 5% CO_2 overnight before treatment.

RNA isolation and Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen), according the manufacturer's instructions. Reverse transcription was carried out using the High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA). Taqman primer-probe sets for mouse Nur77, c-Raf, B-Raf, Egr-1, and β-actin were purchased commercially (Applied Biosystems). Amplification was performed under standard conditions using the ABI Prism 7500 Sequence Detection System. Transcript levels were normalized to corresponding levels of β-actin, and were calibrated to the control group within each experiment.

Data analysis

Data were analyzed using t-test or One-way Analysis of Variance. Overall differences for t-test and ANOVA were considered significant at $p \leq 0.05$. Post hoc

tests involving comparison of multiple treatments against a control were performed using Dunnett's test. All other post hoc tests were performed using Bonferroni's all pairwise comparisons. Differences in post hoc pairwise comparisons were considered significant at $p \leq 0.05$ per comparison.

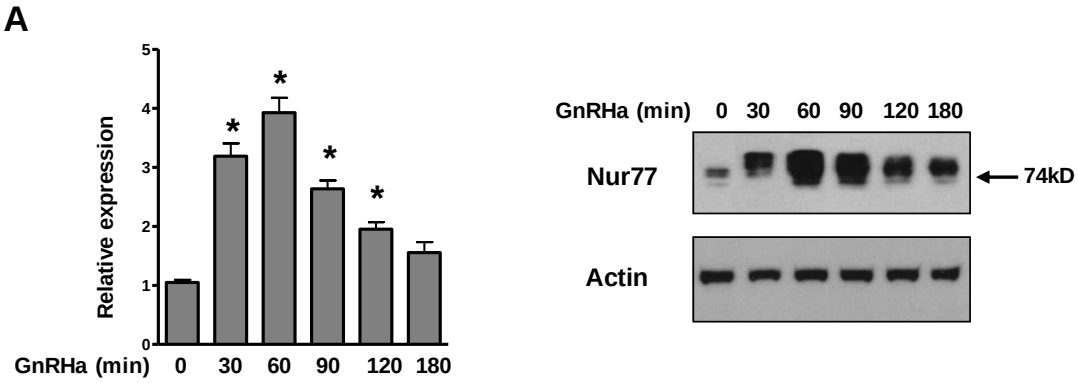
RESULTS

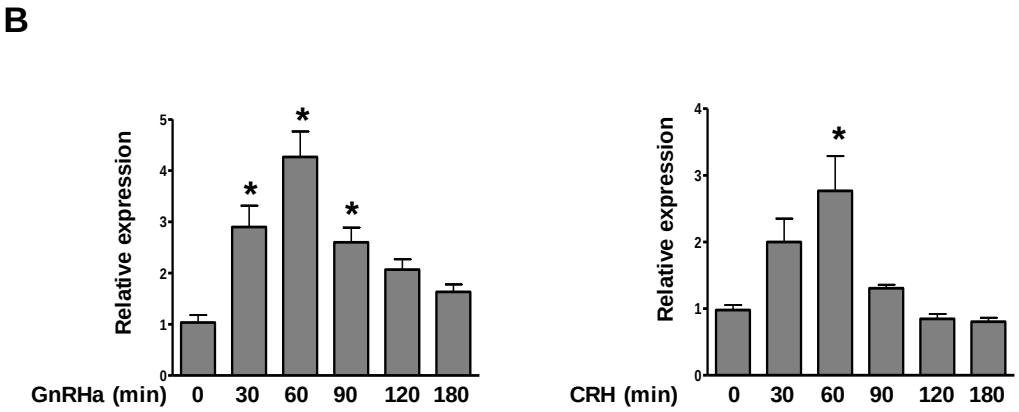
Nur77 is upregulated with immediate early gene kinetics in both αT3-1 cells and primary mouse gonadotropes following GnRHa stimulation

Nur77 has been shown to be expressed in αT3-1 cells; however, its responsiveness to GnRH stimulation has not been reported in this cell line (21). To address this, αT3-1 cells were exposed to the GnRH agonist buserelin over a 3-hour time course, and Nur77 upregulation was examined at the mRNA level by qPCR and at the protein level by immunoprecipitation and immunoblotting. Nur77 transcripts were approximately 4-fold upregulated after one hour of GnRHa stimulation and returned to baseline levels within 4 hours (Figure 4.1A, left panel and data not shown). Nur77 protein levels were also robustly upregulated by GnRHa reaching maximal expression at approximately one hour of GnRHa stimulation (Figure 4.1A, right panel). Induced Nur77 immunoreactivity appeared as multiple closely spaced bands on the immunoblot suggesting posttranslational modification of the induced protein. Consistent with previous reports, phosphatase treatment of the immunoprecipitates resolved the Nur77 immunoreactivity into a more uniform size of approximately 74 kD (data not shown). These results identify Nur77 as a GnRH-responsive immediate early gene in αT3-1 cells and indicate that GnRHa-induced signaling activity in these cells leads to both induction and multi-site phosphorylation of Nur77.

Nur77 has been shown to be induced by CRH in pituitary corticotropes where it plays a role in the transcriptional upregulation of the pro-opiomelanocortin (POMC)

Figure 4.1. Upregulation of Nur77 in αT3-1 and primary pituitary cells following stimulation with GnRHa. A. αT3-1cells were exposed to the GnRH agonist buserelin for the indicated times and Nur77 mRNA levels were measured by qPCR (left panel). Bars represent mean +/- SEM of 3 experimental units. Asterices indicate significant differences ($p \leq 0.05$) from the zero time point. The experiment was repeated 3 times with similar results. B. Upregulation of Nur77 protein was also examined by immunoblotting (right panel). Actin is shown as a lane loading control. B. Whole mouse pituitaries were dispersed into primary culture. Following serum starvation, cultures were exposed to either GnRHa (left panel) or CRH (right panel) for the indicated time courses. Nur77 mRNA levels were measured within each experimental unit by qPCR. For each graph, bars represent mean +/- SEM for 6 replicates representing pooled results from 2 separate experiments. Asterices indicate significant differences from the 0-time point at $p \leq 0.05$.


A
Relative expression
GnRHa (min) 0 30 60 90 120 180
GnRHa (min) 0 30 60 90 120 180
Nur77
74kD
Actin


B
Relative expression
GnRHa (min) 0 30 60 90 120 180
Relative expression
CRH (min) 0 30 60 90 120 180

gene (24). The corticotrope thus represents an established model of Nur77 upregulation induced by activation of a G-protein coupled receptor. To determine whether Nur77 is induced by GnRHa in primary mouse gonadotropes, and to compare its induction kinetics between gonadotropes and corticotropes, whole mouse pituitaries were dispersed into primary culture and then stimulated with either vehicle, GnRHa, or CRH. GnRHa stimulation led to rapid and significant (approximately 4-fold) upregulation of Nur77 transcript levels within mixed primary pituitary cell cultures, the kinetic profile of which was highly similar to that observed in the αT3-1 cell line (Figure 4.1B, left panel). CRH stimulation led to a significant, but more modest (approximately 3-fold) increase in Nur77 transcript levels, with maximal expression observed following 60 minutes of agonist exposure (Figure 4.1B, right panel). CRH-induced Nur77 transcript levels returned to baseline within 120 minutes of agonist exposure, while the transcript upregulation induced by GnRHa was more sustained, remaining significantly elevated above baseline levels for at least 3 hours following agonist exposure (Figure 4.1B).

Nur77 is expressed in mouse pituitary gonadotropes in vivo

To assess the expression and subcellular localization of Nur77 in primary mouse gonadotropes, we examined pituitary sections from randomly cycling female mice using fluorescence immuno-colabeling of Nur77 and LHβ. Nur77 was expressed in many cells throughout the anterior pituitary parenchyma and colocalized extensively with LHβ. Within LHβ-positive gonadotropes, Nur77 was localized predominantly to the nucleus (Figure 4.2).

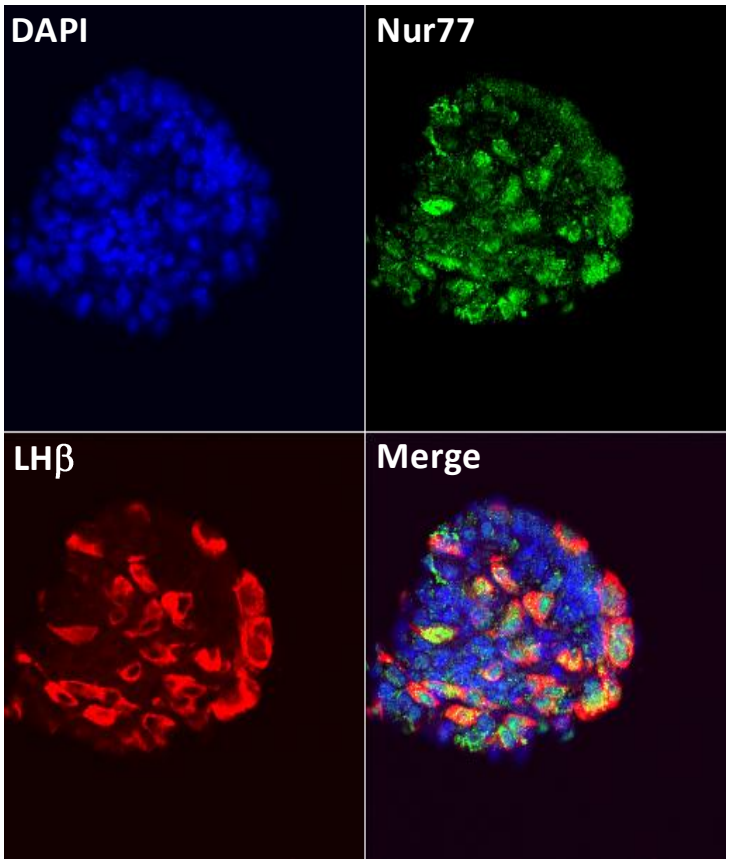
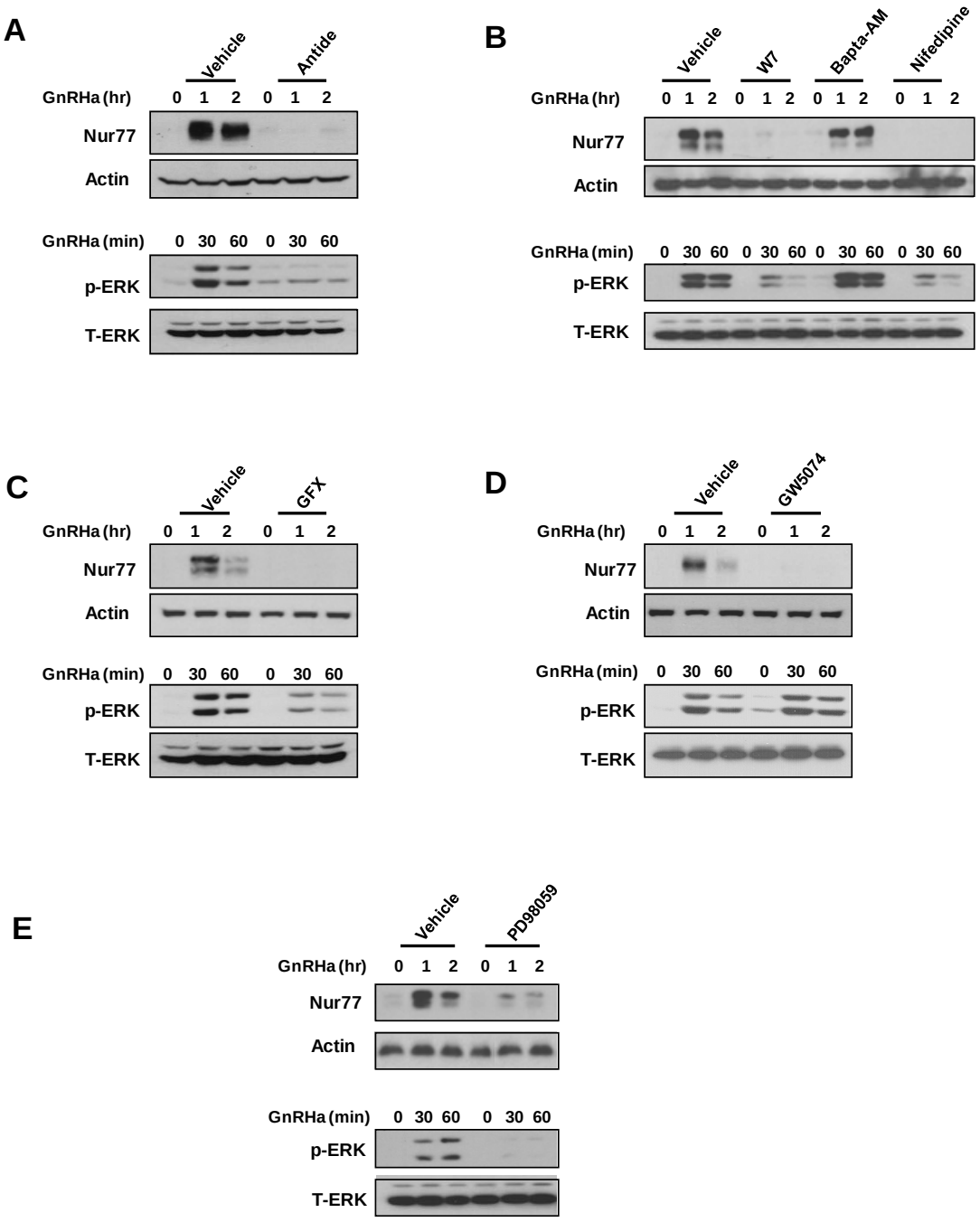


Figure 4.2. Nuclear localization of Nur77 in mouse pituitary gonadotropes in vivo. Expression of Nur77 in gonadotropes in vivo was examined by immunofluorescent co-labeling of sections on mouse pituitary using a FITC-conjugated antibodies against Nur77 and Texas Red-conjugated antibodies against LHβ. Individual labelings of Nur77 and LHβ, and well as simultaneous visualization of both wavelengths (merge) and DAPI are shown. White arrow indicates a gonadotrope that coexpresses LHβ and Nur77 within the cytosolic and nuclear compartments respectively.

GnRHa-induced upregulation of Nur77 in αT3-1 cells is dependent on the classical calcium-PKC-ERK signaling pathway

Having established Nur77 as a GnRH-responsive immediate early gene in αT3-1 cells as well as differentiated gonadotropes, we next employed a cadre of pharmacological inhibitors of specific signaling events known to be induced by GnRH, and analyzed their effects on both GnRHa-induced Nur77 expression, and ERK activation in these cells. As expected, pretreatment of cells with the GnRHR antagonist antide completely abrogated both ERK activation and Nur77 upregulation following GnRHa treatment (Figure 4.3A). Inhibition of PKC isozymes with the agent GF109203X (GFX) similarly attenuated both Nur77 induction and ERK activation (Figure 4.3B). Inhibition of both calmodulin and calcium flux through L-type voltage gated calcium channels with the compounds W7 and nifedipine, respectively, completely blocked GnRH-induced Nur77 expression while reducing, but not abolishing, the ERK response (Figure 4.3C). In contrast, inhibition of intracellular calcium fluxes with the intracellular calcium chelator Bapta-AM appeared to decrease Nur77 induction slightly, while having no effect on ERK activation (Figure 4.3C). Pretreatment of cells with GW5074, an inhibitor of the catalytic activity of c-Raf, abolished the Nur77 response to GnRH stimulation (Figure4.3D). Surprisingly, however, c-Raf inhibition had no effect on GnRH-induced activation of ERK1/2 (Figure 4.3D). Finally, inhibition of MEK1/2 with the specific inhibitor PD98059 significantly decreased GnRHa-induced Nur77 upregulation while predictably abolishing ERK activation (Figure 4.3E). Together these results reinforce the requirement for ERK signaling for GnRH-induced Nur77 upregulation in αT3-1 cells, and provide further demonstration of the roles of PKC, extracellular calcium influx, and calmodulin in mediating the effects of GnRH action on this immediate early gene.

Figure 4.3. Pharmacological analysis of Nur77 upregulation in αT3-1 cells. Serum-starved αT3-1 cells were pretreated for 30 minutes with either vehicle or the indicated pharmacological agents for 30 minutes. Cells were then exposed to a time course (either 1 hour or 2 hours, as indicated) of GnRHa. Lysates from cells exposed to a 2-hour time course of GnRHa were analyzed by immunoprecipitation followed by immunoblotting for Nur77 upregulation (upper portion of panels). Actin levels within the lysates are shown as a control for the input of the immunoprecipitation. Lysates from cells treated in parallel over a 1-hour time course were analyzed by imunoblot for levels of activated ERK (p-ERK, lower portion of panels). Total ERK immunoreactivity is shown as a lane loading control for these samples. Each experiment was repeated at least 3 times with similar results.

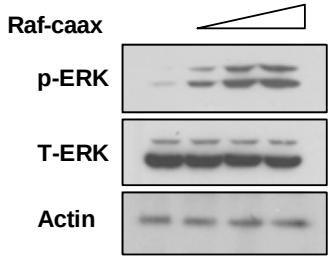

A
Vehicle
Antide
GnRHa (hr)
0 1 2 0 1 2
Nur77
Actin
GnRHa (min)
0 30 60 0 30 60
p-ERK
T-ERK
B
Vehicle
W7
Bapta-AM
Nifedipine
GnRHa (hr)
0 1 2 0 1 2 0 1 2 0 1 2
Nur77
Actin
GnRHa (min)
0 30 60 0 30 60 0 30 60 0 30 60
p-ERK
T-ERK
C
Vehicle
GFX
GnRHa (hr)
0 1 2 0 1 2
Nur77
Actin
GnRHa (min)
0 30 60 0 30 60
p-ERK
T-ERK
D
Vehicle
GW5074
GnRHa (hr)
0 1 2 0 1 2
Nur77
Actin
GnRHa (min)
0 30 60 0 30 60
p-ERK
T-ERK
E
Vehicle
PD98059
GnRHa (hr)
0 1 2 0 1 2
Nur77
Actin
GnRHa (min)
0 30 60 0 30 60
p-ERK
T-ERK

Our pharmacological data indicate that PKC activation, calcium, calmodulin, and ERK signaling are required for GnRH-induced upregulation of Nur77 in αT3-1 cells. However, in this cell line, as in other systems, ERK activation itself has been shown to be dependent on PKC, calcium, and calmodulin (1, 27, 32-34). Therefore, to determine if ERK signaling is sufficient for GnRHa-induced transcriptional upregulation of Nur77 in αT3-1 cells, we utilized an expression vector encoding c-Raf fused to the CAAX membrane-targeting sequence from the small GTP-binding protein ras (Raf-CAAX). Raf-CAAX associates constitutively with the plasma membrane where it drives sustained and high level activation of the ERK pathway independent of extracellular stimuli.

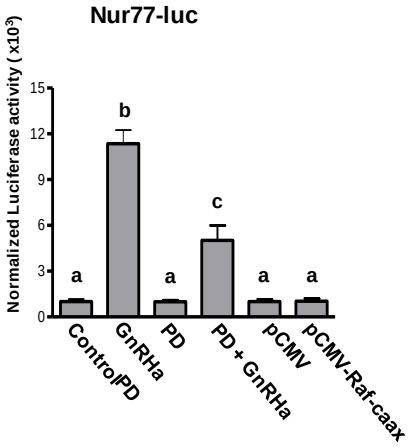
Overexpression of Raf-CAAX in αT3-1 cells led to robust and sustained phosphorylation of ERK1/2 (Figure 4.4A). Cells were then transfected with a luciferase reporter containing 1,566 bp of the mouse Nur77 proximal promoter (Nur77-luc). In addition, some cells were transfected with either pCMV-Raf-CAAX or the empty control vector (pCMV). Following transfection, cells were exposed to either vehicle or the MEK inhibitor PD98059, followed by treatment with either vehicle or GnRHa as indicated. GnRHa stimulation led to robust activation of the Nur77 luciferase reporter; however, this effect was significantly reduced by pharmacological inhibition of ERK signaling (Figure 4.4B, left panel). Significant increases in Nur77 luciferase reporter activity were not observed following transfection of Raf-CAAX alone (Figure 4.4B, left panel). To verify the ability of Raf-CAAX to drive a transcriptional response in these cells, we repeated the experiment using a c-Fos luciferase reporter (c-Fos-luc). GnRHa induced a significant increase in the activity of the Fos-luc reporter which was largely abrogated by inhibition of MEK1/2. Raf-CAAX also induced a significant increase in

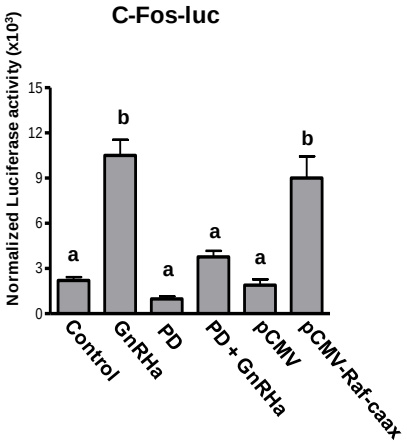
Figure 4.4. ERK signaling is required, but not sufficient, for GnRH-induced transcriptional upregulation of Nur77 in αT3-1 cells. A. αT3-1 cells were transfected for 4 hours with increasing quantities (0.25, 1.0, or 2.5 ug) of Raf-CAAX expression vector. After an additional 4 hour incubation in complete media, cell lysates were analyzed by immunoblot for levels of activated ERK1/2 (p-ERK). Total ERK (T-ERK) and actin immunoreactivity are shown as lane loading controls. B. αT3-1 cells were transfected with a Nur77-luciferase reporter (Nur77-luc, left panel) or a c-Fos-luciferase reporter (C-Fos-luc, right panel). In addition, some cells were transfected with empty control vector or a Raf-CAAX expression vector to drive hormone-independent activation of the ERK pathway. Cells were pretreated or not as indicated with the MEK inhibitor PD90859 (PD), and/or the GnRH agonist buserelin. Bars represent mean +/- SEM normalized luciferase activity of 3 experimental units. Bars with different letter designations represent mean values that are statistically significantly different at $p \leq 0.05$. C. αT3-1 cells were pretreated or not with PD98059, or were transfected with empty vector or Raf-CAAX as indicated, and were then treated with either vehicle or GnRHa as in (A). Bars represent mean +/- SEM endogenous Nur77 (left panel) or Egr1 (right panel) transcript levels as measured by qPCR. Bars with different letter designations represent mean values that are statistically significantly different at $p \leq 0.05$. These experiments were repeated at least 3 times with similar results.

A

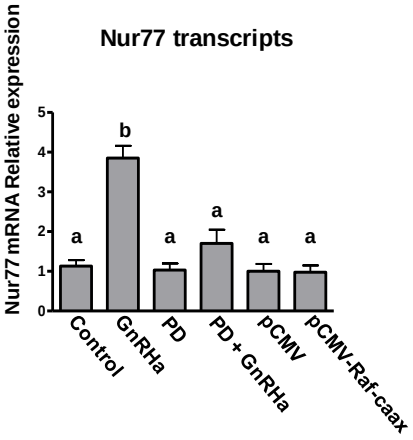

Raf-caax
p-ERK
T-ERK
Actin

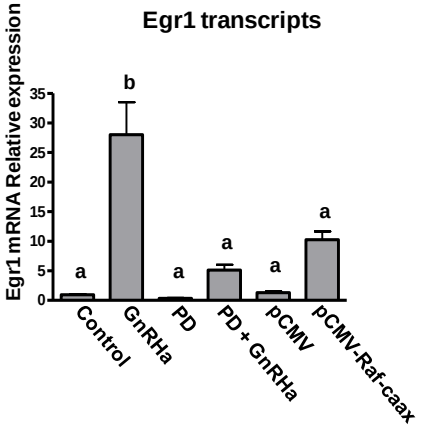
B


Nur77-luc
Normalized Luciferase activity (x10³)
15
12
9
6
3
0
a
b
a
c
a
a
Control
GnRHa
PD
PD + GnRHa
pCMV
pCMV-Raf-caax


C-Fos-luc
Normalized Luciferase activity (x10³)
15
12
9
6
3
0
a
b
a
a
a
b
Control
GnRHa
PD
PD + GnRHa
pCMV
pCMV-Raf-caax

C


Nur77 transcripts
Nur77 mRNA Relative expression
5
4
3
2
1
0
a
b
a
a
a
a
Control
GnRHa
PD
PD + GnRHa
pCMV
pCMV-Raf-caax


Egr1 transcripts
Egr1 mRNA Relative expression
35
30
25
20
15
10
5
0
a
b
a
a
a
a
Control
GnRHa
PD
PD + GnRHa
pCMV
pCMV-Raf-caax

Fos-luc activity, demonstrating the ability of Raf-CAAX to activate an ERK-responsive promoter (Figure 4.4B, right panel).

To assess the effect of ERK signaling on the endogenous *Nur77* gene in αT3-1 cells, we treated cells as described above, and then measured *Nur77* transcript levels by qRT-PCR. The inductive effect of GnRHa on *Nur77* transcript levels was blocked by pharmacological inhibition of the ERK pathway (Figure 4.4C, left panel). Consistent with our observations on the Nur77 luciferase reporter, overexpression of Raf-CAAX had no effect on endogenous *Nur77* transcript levels in these cells (Figure 4.4B, left panel). To assess the ability of Raf-CAAX to induce transcriptional upregulation of an endogenous gene in these cells, we also measured transcript levels of the ERK responsive immediate early gene *Egr1* in these samples. Treatment with GnRHa led to an approximately 27-fold increase in *Egr1* transcript levels within one hour; however, this induction was largely blocked by inhibition of MEK1/2 (Figure 4.4C, right panel). Raf-CAAX led to a more modest (approximately 10-fold) elevation in *Egr1* transcript levels over empty vector treated controls. This effect did not achieve statistical significance in the context of a one-way ANOVA with post-hoc all pairwise comparisons; however, this likely reflects type II error. Collectively, these results indicate that ERK signaling is required, but not sufficient, for GnRHa-induced transcriptional upregulation of *Nur77* in αT3-1 cells.

Pharmacological inhibition of c-Raf does not inhibit GnRHa-induced activation of ERK1/2 in αT3-1 cells

Our pharmacological experiments yielded the unexpected finding that phosphorylation of ERK1/2 is unaffected by pretreatment of αT3-1 cells with the c-Raf inhibitor GW5074. This raises the intriguing hypothesis that GnRH-induced

activation of the ERK pathway may in fact be independent of c-Raf catalytic activity. To test this further, we treated both αT3-1 cells and NIH-3T3 mouse fibroblasts with either PD98059 or a range of doses of GW5074, and evaluated the response of the ERK pathway to treatment with either GnRHa or the classical receptor tyrosine kinase ligand epidermal growth factor (EGF). We chose the NIH-3T3 cell line as a control for this experiment since these cells represent a model in which growth factor induced, c-Raf-dependent ERK activation was initially characterized (35). Predictably, PD98059 abolished the ERK response to both GnRHa and EGF in αT3-1 and 3T3 cells, respectively (Figure 4.5). Pretreatment of αT3-1 cells with GW5074 over a dose range of 1-10 uM had no effect on GnRHa-induced ERK activation (Figure 4.5, left panel). Consistent with previous reports (36), exposure of αT3-1 cells to higher concentrations of GW5074 (>10 uM) led to GnRHa-independent ERK activation (data not shown). In contrast, EGF-induced ERK activation in NIH 3T3 cells was markedly inhibited by GW5074 at doses in excess of 1 uM (Figure 4.5, right panel). These results confirm the activity of this pharmacological agent, and lend further support to the hypothesis that GnRHa-induced ERK activation does not require the catalytic activity of c-Raf.

Neither overexpression of a dominant negative catalytic mutant of c-Raf, nor siRNA-mediated knockdown of c-Raf expression, inhibits GnRHa-induced activation of ERK1/2 in αT3-1 cells

The inhibitor GW5074 has been a useful pharmacological screening tool to assess the role of c-Raf in various cellular processes. However, like many pharmacological agents, its specificity can be difficult to verify in any given experimental setting. To circumvent this difficulty, and further examine the role of

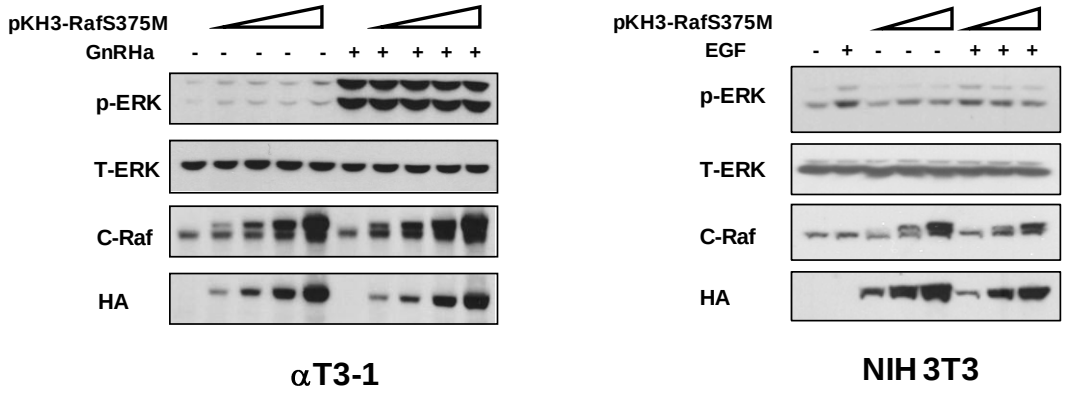
Figure 4.5. Comparative effects of c-Raf inhibition on GnRH- and EGF-induced ERK activation in αT3-1 and NIH-3T3 cells. Serum-starved αT3-1 cells (left panel), or NIH-3T3 cells (right panel) were pre-treated for 30 minutes as indicated with either vehicle, PD98059, or increasing concentrations (1, 5, or 10 uM) of the c-Raf inhibitor GW5074. Cells were then exposed to either vehicle, GnRHa or EGF for 10 minutes. Cell lysates were analyzed by immunoblot for levels of activated ERK (p-ERK). Total ERK immunoreactivity (T-ERK) is shown as a lane loading control. Experiments were performed at least three times with similar results.

c-Raf in mediating GnRH-induced ERK activation, we employed the complementary strategies of dominant-negative c-Raf overexpression, and c-Raf knockdown using RNA interference. αT3-1 cells and NIH 3T3 cells were transiently transfected with increasing doses of an expression construct encoding an HA-tagged catalytically inactive form of c-Raf (Raf-S375M). Cells were then stimulated with either vehicle or GnRHa (αT3-1 cells), or vehicle or EGF (NIH 3T3) cells and ERK phosphorylation was examined by immunoblot. Dose-dependent expression of the recombinant protein was verified by immunoblot against c-Raf and HA. Consistent with our pharmacological data, GnRHa-induced ERK activation in αT3-1 cells was unaffected by overexpression of Raf-S375M at any dose (Figure 4.6A, left panel). In contrast, even within this transient transfection paradigm, modest inhibition of EGF-induced ERK activation in NIH 3T3 cells was observed at the highest dose of Raf-S375M overexpression (Figure 4.6A, right panel).

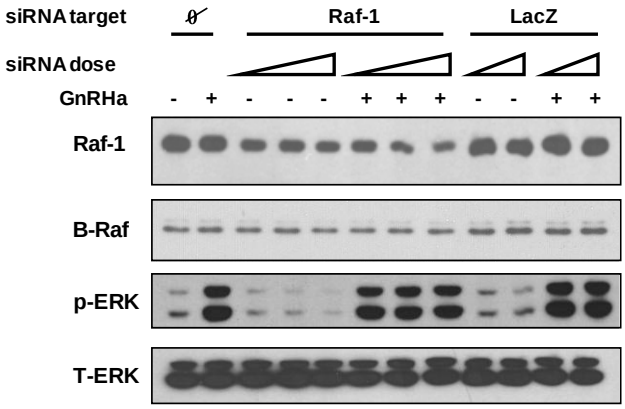
In some cellular models, RNAi-mediated decreases in cellular c-Raf protein levels have been shown to cause proportional decreases in ERK activation following agonist treatment (37). To determine whether suppression of c-Raf protein in αT3-1 cells affects the ability of GnRHa to activate the ERK pathway, we transiently transfected cells with increasing doses of siRNA's against either c-Raf or LacZ mRNA. Cells were then stimulated with either vehicle or GnRHa and ERK phosphorylation was examined by immunoblot. Levels of c-Raf, as well as B-Raf were also assessed by immunoblot to verify specificity of knockdown of the target Raf isoform. c-Raf siRNA resulted in significant reductions in cellular c-Raf protein, with no comparable reduction in levels of B-Raf (Figure 4.6B). GnRHa-induced ERK activation was again unaffected by the decrease in c-Raf protein levels. Together, these data support the conclusion that c-Raf is not required for GnRH-induced activation of the ERK pathway in gonadotropes.

Figure 4.6. Effects of c-Raf dominant negative overexpression and siRNA-mediated c-Raf knockdown on GnRH-induced ERK activation in αT3-1 cells. A. αT3-1 cells (left panel) or NIH-3T3 cells (right panel) were transfected with increasing doses of an expression vector encoding an HA-tagged dominant-negative c-Raf (pKH3-Raf-S375A). Following transfection, cells were serum-starved, and then stimulated with either vehicle or GnRHa for 10 minutes. Cell lysates were analyzed by immunoblot for levels of activated ERK (p-ERK). Total ERK immunoreactivity (T-ERK) is shown as a lane loading control. Lysates were also analyzed for immunoreactivity against Raf-1 and the HA epitope tag. B. αT3-1 cells were transfected with increasing doses of siRNA targeting either c-Raf, or the LacZ coding sequences. Following transfection, cells were serum-starved, and then stimulated with either vehicle or GnRHa for 10 minutes. Cell lysates were analyzed by immunoblot for levels of activated ERK (p-ERK). Total ERK immunoreactivity (T-ERK) is shown as a lane loading control. Lysates were also analyzed for immunoreactivity against B-Raf to assess isoform specificity of the anti-c-Raf siRNA. These experiments were performed at least three times with similar results.

A


pKH3-RafS375M
GnRHa
- - - - - + + + + +
p-ERK
T-ERK
C-Raf
HA
αT3-1
pKH3-RafS375M
EGF
- + - - - + + +
p-ERK
T-ERK
C-Raf
HA
NIH 3T3

B


siRNA target
Ø
Raf-1
LacZ
siRNA dose
GnRHa
- + - - - + + + - - + +
Raf-1
B-Raf
p-ERK
T-ERK

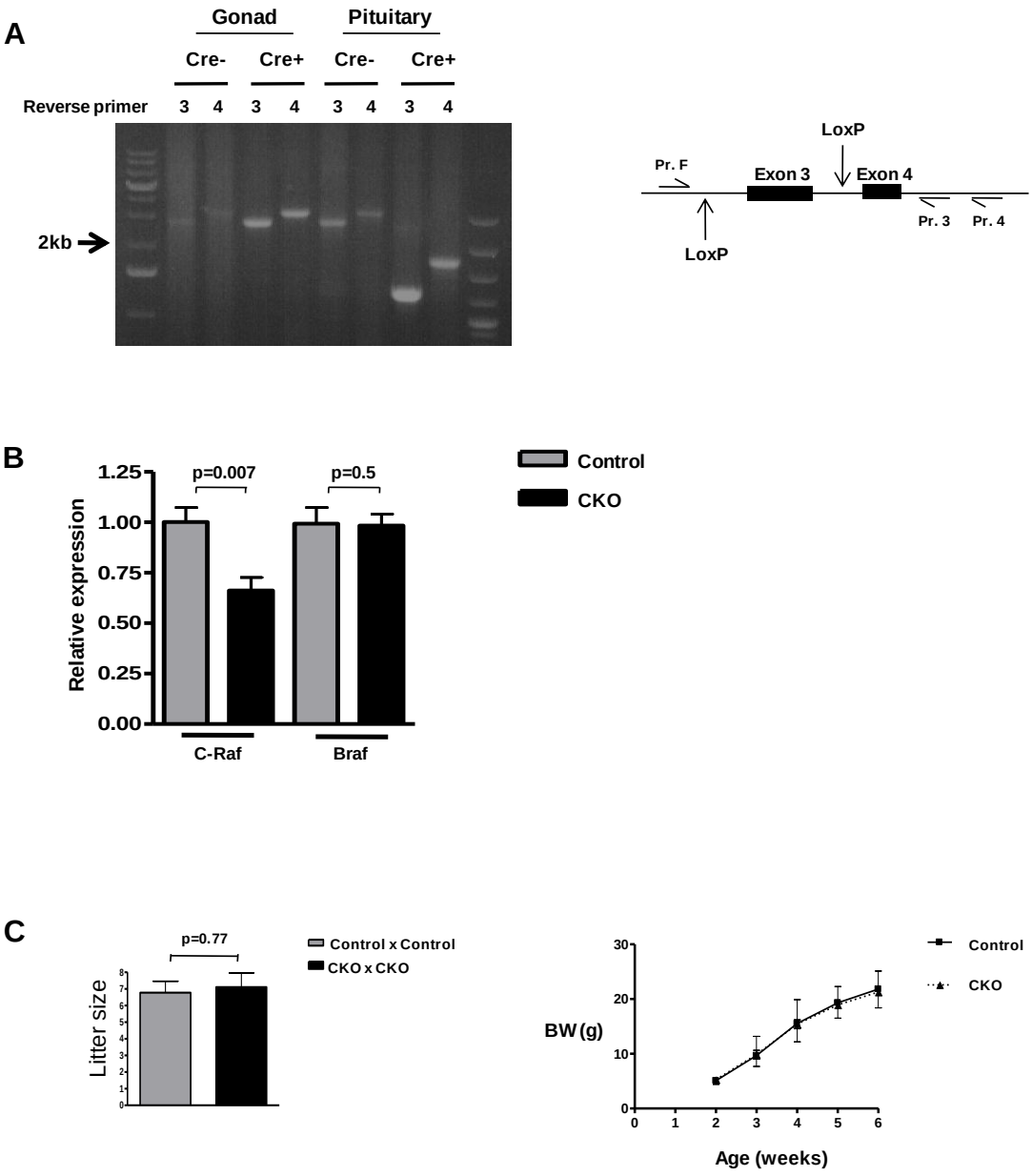
Generation and validation of a pituitary specific c-Raf knockout mouse.

The data presented here support use of the αT3-1 cell line as a model for study of the mechanisms of GnRH-induced Nur77 expression in the gonadotrope. However, to further corroborate our results, we sought to develop an in vivo model by which to compare the roles of c-Raf and ERK activity in mediating the Nur77 transcriptional response to GnRH. Previously, we described generation of a transgenic mouse model with pituitary targeted ablation of ERK1 and 2 that resulted in female infertility due to LH deficiency (30). Using a similar genetic approach, we generated mice with pituitary-targeted conditional ablation of c-Raf (c-Raf conditional knockout, c-Raf CKO). We examined a range of physiological parameters in c-Raf CKO animals, including fertility, to establish the role of c-Raf for gonadotrope function in vivo, and then specifically asked whether c-Raf is required for GnRH-induced transcriptional upregulation of Nur77 in vivo.

Mice homozygous for a floxed mutation at the *c-Raf* locus (*c-Raf*$^{fl/fl}$), were crossed with *αGSU:Cre* mice, in which Cre recombinase is expressed under the regulatory control of a 4.6 kb fragment of the murine αGSU promoter. Cre-dependent recombination at the *c-Raf* locus was assessed by PCR in *c-Raf*$^{fl/fl}$,*CRE*$^{+}$ and *c-Raf*$^{fl/fl}$,*CRE*$^{-}$ animals using primers flanking the floxed region of the *c-Raf* locus (Figure 4.7A, schematic). Products indicating excision of the floxed region of the *c-Raf* gene were only produced from pituitary genomic DNA of Cre^{+} individuals, indicating tissue-specific susceptibility of the *c-Raf* locus to Cre-mediated recombination (Figure 4.7A, left panel).

To further verify this transgenic model, we compared pituitary transcript levels of *c-Raf* in *c-Raf*$^{fl/fl}$,*CRE*$^{+}$ and *c-Raf*$^{fl/fl}$,*CRE*$^{-}$ animals using quantitiative PCR (qPCR). Transcript levels of *c-Raf* were significantly lower in Cre-expressing animals as

Figure 4.7. Validation of the pituitary targeted c-Raf conditional knockout mouse. A. Cre-mediated recombination at the *c-Raf* locus within the pituitary was detected by PCR using genomic DNA from the specified tissues. For each sample, the forward primer designated 'Pr. F' was paired individually with reverse primers labeled 'Pr. 3' and 'Pr. 4' spanning the floxed genomic region as indicated in the schematic. Lanes are labeled with the specified reverse primer used for the reaction. Molecular weight marker is shown in the left lane. Equivalent results were obtained from males and females. B. Whole pituitary transcript levels of c-Raf and B-Raf were measured by qPCR in control and CKO animals. Bars represent mean +/- SEM from 5 animals per group. Means were compared by 2-tailed t-test with p values indicated. C. Litter sizes from matings between control or CKO animals were compared by 2-tailed t-test (left panel). Bars represent mean +/- SEM of 9 litters per group. Body weights were compared between control and CKO animals from 2 weeks through 6 weeks of age (right panel). Points represent mean +/- SEM of body weights from the same 5 animals from each group. Data are shown for females; however similar results were obtained for males.


A
Gonad
Pituitary
Cre-
Cre+
Cre-
Cre+
Reverse primer
3 4 3 4 3 4 3 4
2kb
LoxP
Pr. F
Exon 3
Exon 4
Pr. 3
Pr. 4
LoxP
B
Relative expression
1.25
1.00
0.75
0.50
0.25
0.00
p=0.007
p=0.5
C-Raf
Braf
Control
CKO
C
Litter size
p=0.77
Control x Control
CKO x CKO
BW (g)
Age (weeks)
Control
CKO

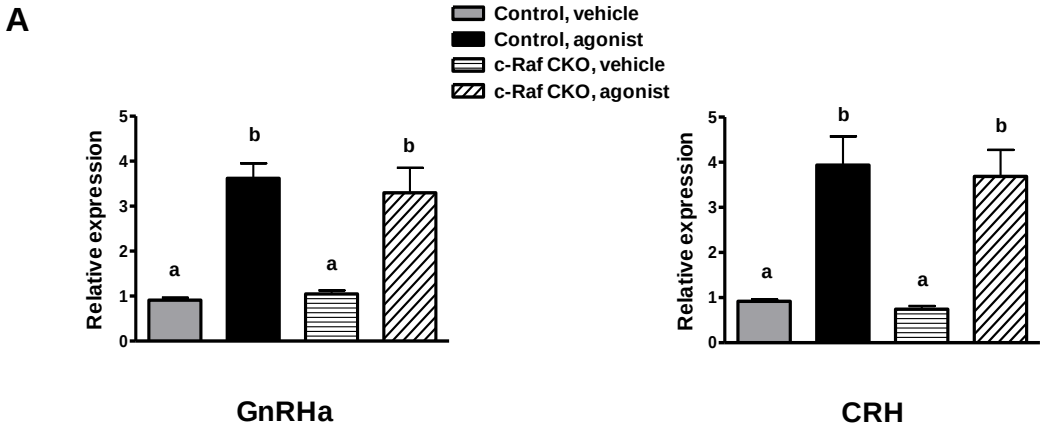
compared with Cre-non-expressing controls (Figure 4.7B, left panel). In contrast, *B-Raf* transcript levels were not significantly different between c-Raf CKO and control mice (Figure 4.7B). The degree of reduction in c-Raf transcript levels is consistent with the fact that only a minority of cells within the pituitary express the αGSU and would be expected to be rendered c-Raf deficient in this model. These data further support the fidelity of the genetic perturbation in this model.

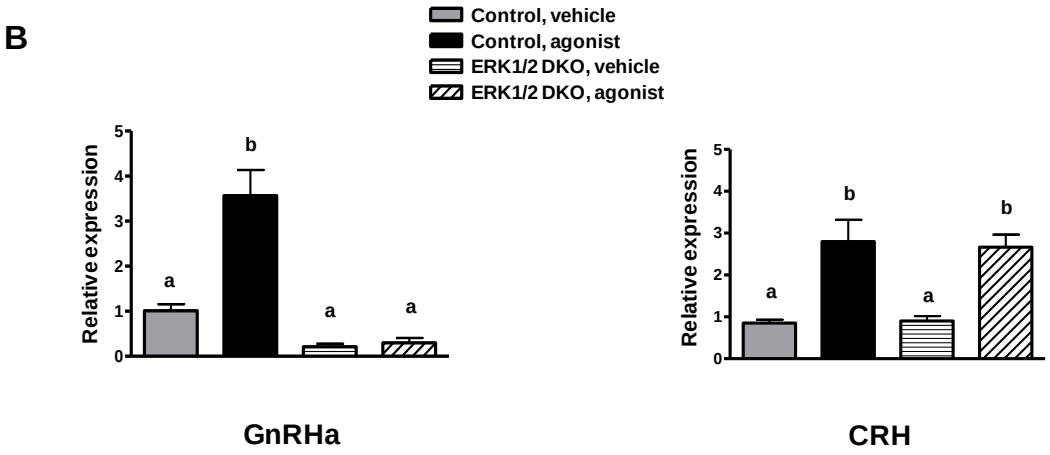
Pituitary-specific c-Raf null males and females were viable and fertile. They were born at expected Mendelian frequencies, grew at the same rate as control littermates and were grossly and histologically indistinguishable from either control littermates or wild type mice at adulthood (Figure 4.7C, right panel and data not shown). Litter sizes from matings between control animals were not significantly different from litter sizes from matings between CKO animals (Figure 4.7C, left panel). These observations support the conclusion that c-Raf is not required for the function of differentiated gonadotropes in vivo.

Basal and GnRHa-induced transcriptional upregulation of Nur77 in primary mouse gonadotropes requires ERK signaling but is independent of c-Raf

To address the requirements for c-Raf and ERK activity for GnRH-induced expression of Nur77 in vivo, we dispersed whole pituitaries from mice with pituitary deficiency of c-Raf, ERK1/2, or their respective control littermates into primary culture and stimulated the cells with either GnRHa or CRH. Nur77 transcript levels were then measured in the cultures by qPCR. In pituitary cell cultures from mice with pituitary deficiency of c-Raf, both GnRHa and CRH led to significant increases in Nur77 transcript levels which were indistinguishable from the response of the controls (Figure 4.8A). CRH stimulation led to significant increases in Nur77 transcript levels

Figure 4.8. GnRHa- or CRH-induced transcriptional upregulation of Nur77 in primary mixed pituitary cell cultures from mice with pituitary-targeted ablation of either c-Raf, or ERK1/2. A. Whole pituitaries from mice with αGSU-CRE-mediated pituitary-specific disruption of c-Raf (c-Raf conditional knockout, c-Raf CKO) were dispersed into primary culture. Following serum starvation, cultures were exposed to either vehicle, and GnRHa or CRH for 60 minutes. Nur77 mRNA levels were measured within each experimental unit by qPCR. Bars represent mean +/- SEM for 9 replicates representing pooled results from 3 separate experiments. Bars with different letter designations represent mean values that are statistically significantly different at $p \leq 0.05$. B. Whole pituitaries from mice with pituitary-specific compound ablation of ERK1 and 2 (ERK1/2 double knockout, ERK1/2 DKO) were dispersed into primary culture and treated as in (A). Bars represent mean +/- SEM for 6 replicates representing pooled results from 2 separate experiments. Bars with different letter designations represent mean values that are statistically significantly different at $p \leq 0.05$.


A
Control, vehicle
Control, agonist
c-Raf CKO, vehicle
c-Raf CKO, agonist
Relative expression
0
1
2
3
4
5
a
b
a
b
GnRHa
CRH


B
Control, vehicle
Control, agonist
ERK1/2 DKO, vehicle
ERK1/2 DKO, agonist
Relative expression
0
1
2
3
4
5
a
b
a
a
b
a
b
GnRHa
CRH

in both control mice and mice with pituitary deficiency of ERK1/2 (Figure 4.8B, right panel). In contrast, while GnRHa stimulation induced significant increases in Nur77 transcript levels in control mice, this inductive effect of GnRHa was completely blocked in ERK deficient pituitary cells (Figure 4.8B, left panel). Together, these results establish Nur77 as a GnRH-responsive, ERK-dependent immediate early gene in the gonadotrope, and indicate further that c-Raf is not an obligate intermediary in the GnRH-ERK signaling pathway in vivo.

DISCUSSION

Using both cellular and in vivo models, we show here that the orphan nuclear receptor Nur77 is rapidly and robustly upregulated in gonadotropes following GnRH stimulation. Activation of the ERK signaling pathway is required, but not sufficient for Nur77 induction both in the αT3-1 gonadotrope cell model, and in vivo. Consistent with the observation that c-Raf kinase is not required for GnRH-induced transcriptional upregulation of Nur77, our data reveal further that c-Raf is not the primary MAPKKK of the ERK pathway within these cells. In these studies, the kinetics and magnitude of Nur77 induction were highly similar between the αT3-1 cell line and primary pituitary gonadotropes. This contrasts with a recent report demonstrating sustained expression of Nur77 in the LβT2 cell line following GnRH stimulation (38). The overall correspondence between αT3-1 cells and primary gonadotropes supports the validity of this cell line as a model for study of GnRH-induced Nur77 expression in the gonadotrope.

While perhaps controversial within the context of GnRH signaling, our observations on the c-Raf independence of ERK pathway activation in the gonadotrope are consistent with many contemporary reports on the cellular functions of c-Raf (39-41). C-Raf dependent activation of the ERK pathway remains a widely

accepted paradigm, yet a growing body of evidence indicates that MEK kinase activity is not a primary function of c-Raf. Gene ablation studies showed that c-Raf deficient mice die at approximately e10.5 of gestation due to widespread apoptosis; however, growth factor induced ERK activation remained unimpaired in c-Raf-deficient embryonic stem cells, suggesting that the developmental defect in these mice was unrelated to the loss of a critical MEK kinase (42). Moreover, the lethal phenotype in the c-Raf deficient mouse was rescued by reintroduction of a catalytically inactive form of c-Raf showing that the requirement for c-Raf during development is independent of its MEK kinase activity (43). Recent reports using conditional targeted gene ablation approaches indicate instead that c-Raf may serve a primary role as a negative regulator of apoptosis in vivo (29, 41, 44, 45). The mechanism underlying downregulation of sensitivity to apoptotic stimuli has been linked to direct c-Raf mediated inhibition of the proapoptotic proteins ASK1 or MST2 (41, 46). In our examination of mice with pituitary targeted ablation of c-Raf, we found no differences in gonadotrope numbers between c-Raf deficient animals and littermate controls (data not shown). Thus, targeted ablation of c-Raf in pituitary gonadotropes appears to have no effect on cellular phenotype. However, gonadotropes are highly differentiated postmitotic cells that do not undergo apoptosis during the course of their functional life. In addition, given the critical importance of these cells for reproductive function, it may be reasonable to speculate that they have evolved robust and multifaceted resistance to apoptotic stimuli. Therefore, the antiapoptotic activity of c-Raf may be dispensible within this specific cell lineage.

Our studies using the compound GW5074 showed that inhibition of c-Raf activity had no effect on either GnRH-induced ERK activation, or on transcriptional upregulation of Nur77 in αT3-1 cells. Interestingly, however, upregulation of Nur77 at the protein level was largely abrogated by c-Raf inhibition. It is possible that this

represents a non-specific effect of the GW5074 compound on translational processes. Alternatively, this observation may point to a role for c-Raf in translational regulation in these cells. Indeed, GnRH-induced upregulation of the immediate early gene c-Fos at the protein level in αT3-1 cells was unaffected by GW5074 pretreatment (S Bliss, unpublished data). Recently, the translational regulator eukaryotic elongation factor 1A (eEF-1A) was identified as a target of c-Raf-mediated phosphorylation (44). Inhibition of c-Raf activity led to a decrease in interferon-induced eEF-1A phosphorylation which accelerated the ubiquitination and proteosomal degradation of eEF-1A. In turn, downregulation of eEF-1A was associated with increased cellular sensitivity to the pro-apoptotic effects of interferon-α (44). This study points to an additional mechanism by which c-Raf may antagonize apoptosis. More broadly, the identification of eEF-1A as a c-Raf substrate discloses an intriguing link between c-Raf catalytic activity and the translational apparatus of the cell. This is consistent with a previous report that indicated that stimulation of cap-dependent translation by GnRH in αT3-1 cells was partially dependent on ras (and presumably, therefore, c-Raf) but was not dependent on ERK signaling (47). Whether c-Raf catalytic activity is uniquely required for GnRH-induced translational upregulation of Nur77 in these cells is currently being investigated.

Our results indicate that upregulation of Nur77 in αT3-1 gonadotropes is dependent on both PKC and calcium. These findings are consistent with previous reports demonstrating calcium and PKC dependence of Nur77 in a variety of cell types (23, 24, 34, 48). In T lymphocytes, an established model in which Nur77 is strongly upregulated following stimulation of the T cell receptor (TCR), calcium and PKC function as parallel signaling pathways with synergistic effects on Nur77 promoter activation (49). PKC increases Nur77 promoter activity by targeting the transcription factor Jun-D. Jun-D binds constitutively to AP-1-like (NAP) sites of the Nur77

promoter and under resting conditions, represses the Nur77 promoter through recruitment of the corepressor Menin and an mSin3A-HDAC complex (50). Activation of PKC leads to phosporylation of Jun-D, dissociation of the Menin-corepressor complex, and recruitment of p300 HAT activity to the promoter (50). Importantly, PKC-dependent phosphorylation of Jun-D in this setting is mediated specifically by the ERK pathway (50). In luteinized ovarian cells, induction of Nur77 by the arachidonic acid metabolite prostaglandin $F_{2\alpha}$ was also shown to be dependent on calcium/calmodulin and ERK1/2-mediated phosphorylation of Jun-D; however, in these cells, ERK activation itself was shown to be dependent on calcium/calmodulin (34). Activation of the ERK pathway has similarly been shown to be dependent on PKC, extracellular calcium/calmodulin in αT3-1 gonadotropes (1, 27, 32, 33). Thus, while the molecular mechanisms of Nur77 promoter activation in gonadotropes await precise clarification, it seems reasonable to hypothesize that ERK-dependent phosphorylation of Jun-D may play a key role. In support of this hypothesis, both the expression and the transcriptional activity of Jun-D were previously shown to upregulated by GnRH in the αT3-1 cell line (51).

Nur77 has been shown to serve a variety of physiological roles in different cells types, but functions most commonly as a transcriptional regulator. Monomeric Nur77 binds to a consensus octanucleotide response element (NGFI-B-response element, NBRE) consisting of sequence AAAGGTCA and may function as a transcriptional activator or repressor (12, 48, 52, 53). Nur77 may also dimerize with other members of the NR4A family (Nor-1 or Nurr1) and bind to a bipartite target DNA motif, the Nur response element (NurRE) (54). We have demonstrated the ability of GnRH stimulation to activate multimers of NBRE and NurRE in αT3-1 cells with equal efficiency (S Bliss and MS Roberson, unpublished observations). Alternatively, Nur77 may dimerize with RXR and bind to a DR5 element in a retinoic

acid-dependent manner (54, 55). A recent genomic analysis revealed 483 genes in the human genome that possess an NBRE within their proximal promoter, highlighting the possibility that Nur77 may exert broad transcriptional regulatory influence in various contexts (56). Clarification of the physiological role(s) of Nur77 has been confounded by the observation that Nur77 deficient mice are viable and fertile and display only relatively subtle phenotypic abnormalities associated with dysregulation of central dopaminergic neuronal pathways (10). However, a high degree of functional redundancy between Nur77 and the closely related NR4A family member Nor-1 may underlie the disparities between reported gene regulatory roles of Nur77 and the lack of overt phenotypic abnormalities in the Nur77 deficient mouse (57, 58). Elucidation of physiologically relevant targets of Nur77 in the gonadotrope will likely require a genetic approach involving tissue-specific compound deletion of both Nur77 and Nor-1.

In conclusion, our data establish Nur77 as an immediate early response gene strongly induced by GnRH in gonadotropes in vivo. Analysis of the signaling activities required for upregulation of Nur77 in these cells demonstrate that activation of the Nur77 promoter is dependent on calcium, PKC, and ERK signaling. In addition, these results indicate that activation of the ERK pathway following GnRH receptor occupancy in gonadotropes is independent of c-Raf, and point to a possible alternative role for c-Raf in regulation of translation in these cells.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The functional organization of the vertebrate HPG axis, and the central role of the gonadotrope in the regulation of reproductive function have been appreciated for several decades (1-5). Historically, experimental studies of HPG axis function have emphasized the use of live animal models in which specific experimental manipulations are linked to relatively broad physiological outcome parameters such as fluctuations in plasma hormone levels, or measureable changes in reproductive behaviors or fertility. This general approach is perhaps best exemplified by the work of Ian Clarke who developed a method of chronic surgical cannulation of the hypophyseal portal vessels in sheep allowing high frequency sampling and biochemical analysis of hypophyseal portal blood (6, 7). This seminal work clarified the importance of the pulsatile nature of hypothalamic GnRH secretion and its link to oscillations in plasma levels of the gonadotropins, as well as the fundamental role of gonadal steroids in negative feedback regulation of the activity of the HPG axis.

In contrast to whole animal physiological approaches to the study of the HPG axis, research into the molecular mechanisms underlying gonadotrope function has relied heavily upon use of the established αT3-1 and LβT2 gonadotrope cell lines (8-16). Certainly, neither the complex pulsatile nature of the GnRH signal, nor the integrated feed-forward or feedback endocrine regulatory mechanisms at play in the living animal can be satisfactorily modeled in these simple cell systems. Nevertheless, over the past 20 years, these cell lines have proven to be useful tools for examination of the molecular basis gonadotrope function. More recently, dramatic expansion of the availability of genetically modified mice has provided an opportunity to integrate these approaches, and to test specific hypotheses regarding the molecular mechanisms underlying gonadotrope responsiveness to GnRH within a physiologically relevant whole animal model. The work described in this dissertation has emphasized such

complementary use of basic cellular and in vivo models in the investigation of the role of ERK signaling in gonadotrope function.

Membrane rafts and the GnRHR

Appropriate cell surface expression of the GnRHR by gonadotropes is obviously essential for GnRH action. Previous work has demonstrated that certain forms of hypogonadotropic hypogonadism may result from aberrant intracellular trafficking and lack of appropriate expression of the receptor (17, 18); however, results published by the lab of Dr. Colin Clay in collaboration with this lab provided the first evidence that GnRHR function may be further regulated at the level of the plasma membrane through its association with cholesterol-enriched membrane microdomains (19). The data presented in Chapter 2 of this dissertation indicate that the biophysical properties of the plasma membrane are important for the ability of the GnRHR to couple to the ERK module, and suggest that membrane rafts may play a role in facilitating the assembly of a plasma-membrane associated GnRHR-ERK signaling complex. The composition of this signaling complex warrants more systematic investigation. Mass spectrometry has been useful for identification of raft-associated proteins as well as for identification of individual components of native protein complexes purified by immunoprecipitation or affinity chromatographic techniques (20-23). Our ability to immunoprecipitate the GnRHR from a low-density membrane environment following cell fractionation suggests that a similar strategy may be useful for elucidation of the composition of signaling complexes that associate with membrane rafts in conjunction with the GnRHR.

It is notable that the constitutive and ligand-independent association of the GnRHR with low-density membrane rafts is atypical of GPCRs generally. Most GPCRs show partial constitutive association with membrane rafts, and in many cases,

agonist treatment causes a significant change in the tendency of a receptor to partition into, or out of, a low-density membrane environment (24, 25). Whether the GnRHR has an intrinsic biophysical preference for a lo membrane environment, or whether its behavior is dependent upon specific protein-protein interactions is not entirely clear; however, domain swap studies indicated that the absence of the C-terminal tail does not itself lead to constitutive raft association of the receptor (19). Ultimately the GnRHR may represent an interesting and unique model for study of the biophysical mechanisms underlying the partitioning of GPCRs into lo lipid environments.

The requirement for ERK signaling for gonadotrope function in vivo

The ability of the GnRHR to activate the ERK pathway and the role of ERK signaling in various aspects of gonadotrope function have been convincingly demonstrated in the αT3-1 and LβT2 gonadotrope cell lines (10, 15, 26-31). The data presented in Chapter 3 corroborate the importance of the ERK pathway for gonadotrope function and establish the ERK pathway a major mediator of the effects of GnRH on LHβ transcription in vivo. However, our results further disclose an interesting gender difference in the requirement for ERK signaling for fertility. The female-specific nature of this requirement has not previously been recognized and highlights value of this genetic approach to the study of the molecular basis of gonadotrope function. Whether the gender-specific nature of the requirement for ERK signaling in the gonadotrope represents simply a differential requirement for LH itself between males and females, or whether there exists a more intrinsic gender difference in the requirement for ERK signaling for *LHβ* transcription is not clear. However, the generation and characterization of this model would appear to provide a useful tool with which to address the mechanisms underlying this gender-specific phenotype.

While our data indicate that the major phenotypic impact of gonadotrope-targeted ERK ablation reflects deficient Egr-1 upregulation and failure of *LHβ* transcription, it is likely that phosphorylation of numerous substrates is impaired in the ERK-deficient gonadotrope. Identification of these substrates promises to shed light on finer aspects of the function and regulation of these cells. Indeed, in agreement with previous work (32), transcriptional upregulation of the α-GSU is impaired in the ERK-deficient gonadotrope, yet the gonadal histology in these animals would indicate that this defect does not result in an overt phenotype. Generation of this model of pituitary ERK deficiency brings identification and in vivo validation of additional and perhaps previously unrecognized ERK substrates and ERK-dependent genes well within reach, through either comprehensive genomic or proteomic techniques, or candidate target approaches.

Nur77 in the gonadotrope

Using a candidate gene approach, we show in Chapter 4 of this dissertation that Nur77 is a component of the immediate early gene program induced by GnRH in the gonadotrope. The hypothesis that Nur77 is a GnRH-induced immediate early gene that plays a role in gonadotrope function evolved from previous observations that this orphan nuclear receptor is upregulated in the LβT2 cell line following GnRH stimulation (33), as well as reports describing the role of Nur77 in activation of the proopiomelanocortin promoter in pituitary corticotropes (34). The data in Chapter 4 constitute the first account of the signaling mechanisms involved in mediating Nur77 upregulation in either the αT3-1 cell line or in differentiated gonadotropes in vivo. Use of our pituitary-specific ERK deficient mice confirms that Nur77 is an ERK dependent GnRH-responsive immediate early gene in vivo.

Our observations regarding the c-Raf independence of GnRH-induced ERK pathway activation raise interesting questions regarding the functional organization of the ERK pathway as well as the role(s) of c-Raf in these cells. Reports in the literature on GnRH signaling frequently cite c-Raf as the canonical upstream MAPKKK of the ERK module in the gonadotrope (29, 30, 32, 35, 36); however scrutiny of this literature reveals little to no experimental evidence to support this notion. GnRH stimulation does appear to induce raid phosphorylation and enhanced catalytic activity of c-Raf in gonadotropes (30, 37). Therefore, it seems reasonable to hypothesize that c-Raf may play some role as a MAPKKK in these cells, perhaps in parallel with separate and sufficient pathway(s) linking the GnRHR to the ERK module. Given the importance of ERK signaling for female fertility, it is perhaps not surprising that multiple redundant mechanisms of GnRH-induced ERK activation may have evolved. As with the pituitary-specific ERK deficient mouse, the pituitary-targeted c-Raf deficient model may provide a useful tool for further investigation of the role of c-Raf in the gonadotrope.

While our data on the upregulation of Nur77 in the gonadotrope provide insight into the signaling events that mediate the induction of this gene, the biological functions of Nur77 in these cells remain to be determined. Interestingly, the proximal promoters of both the *αGSU* and the *GnRHR*contain monomeric Nur77 response sequences (NBRE), making these genes attractive candidates for evaluation as Nur77 targets. Indeed, preliminary observations from this lab indicate that overexpression of recombinant Nur77 in αT3-1 cells led to the modest but significant dose-dependent activation of a luciferase reporter containing a 600 bp fragment of the proximal *GnRHR* promoter (S Bliss, unpublished observations). This poses the intriguing possibility that Nur77 may play a role as a low-level transcriptional activator of this

important gonadotrope gene. Attempts to clarify the role of Nur77 in the gonadotrope are ongoing.

Summary

The ERK signaling pathway is an important component of the intracellular signal transduction systems that mediate the effects of GnRH in the gonadotrope. By combining basic cellular and genetic approaches, the studies presented here broaden our understanding of the importance of ERK signaling for the function of differentiated gonadotropes, and raise new questions regarding the organization of this pathway in these cells. It is hoped that this incremental contribution to the broader field of gonadotrope biology may facilitate the development of more effective strategies for management of reproductive dysfunction.

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